STUDIES ON THE SPARGANUM OF

SPIROMETRA ERINACEI

by

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The work reported in this thesis, except where specifically mentioned, was performed entirely by me.

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(KWA BOO-HOE)

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GENERAL INTRODUCTION

Penetration by a parasite through or into the intestinal wall of its host is a common phenomenon; various examples are seen in all major groups of parasites. In the case of the plerocercoids, or spargana, of pseudophyllidean cestodes (genera Diphyllobothrium and Spirometra), considerable interest has been aroused because these organisms are extremely paratenic and exhibit low host-specificity; they are able to penetrate through the gut of a wide variety of intermediate hosts (see Chapter I for review of life cycle). The remarkable behaviour of this larval cestode implies that it possesses an efficient mechanism for penetration of the intestinal wall.

Wardle and McLeod (1952) noted of the pseudophyllidean sparganum that "The method by which such a soft-bodied organism, lacking anything in the nature of a penetrant mechanism and relying mainly upon its own turgidity, can travel from the alimentary canal of its host to an intermuscular position, or to a position within the layers of the gut wall, is far from clearly understood." By implication Wardle and McLeod probably meant that an obvious "penetrant mechanism" is lacking; it is clear,

however, that some mechanism must exist to account for the sparganum's efficacy in penetration.

There would appear to be three main methods by which parasites are able to penetrate the intestine of their hosts. Firstly, by the use of hooks or teeth a parasite may be able to physically tear through the tissues and thus eventually penetrate the intestine by mechanical destruction of the intestinal wall. Secondly, even without hooks or teeth, a parasite with powerful suckers may be able to cause sufficient initial damage to intestinal tissues so as to permit autolysis by intracellular enzymes and enzymes of the intestinal lumen which would facilitate penetration. Thirdly, a parasite may secrete histolytic enzymes for digesting its way through the intestinal wall of the host. These methods may not necessarily be mutually exclusive, and varying combinations of the three may be utilised by different parasites.

In the case of a sparganum, the absence of hooks or teeth rules out the possibility of the first method. The sparganum is also unlikely to make use of the second method for penetration since it does not possess strong muscular suckers, but weakly developed bothria. Since the sparganum takes only a short time to penetrate through

the intestinal wall, the second method may be thought of as even less likely as an adequate mechanism for penetration. That leaves the third possibility as the most likely mechanism involved; however, it should be stressed that once initial damage is done by the parasite, autolytic effects could play a significant role as well.

Previous authors have suggested that histolytic enzymes may be involved in the mechanism of sparganum penetration (Smyth and Heath, 1970). Although, as far as can be determined, such enzymes have not been previously described in any Spirometrid sparganum, proteolytic enzymes have been identified in preparations (plerocercoids or adults not specified) of Diphyllobothrium latum (Read and Simmons, 1963). In addition, a study by Fischer and Freeman (1969), on Proteocephalus ambloplitis plerocercoids, demonstrated that a large gland in the scolex of the parasite secreted a substance which apparently aided penetration. Furthermore, glands have been described in the scolex of other species of cestodes (see Chapter 3) as well as in the procercoid of Spirometra erinacei (Li, 1929). This present study was therefore concerned with an investigation to determine whether glandular secretions might be the basis of penetration of the gut wall of intermediate hosts

by the spargana (plerocercoids) of Spirometra erinacei.

CHAPTER I

THE SPARGANUM

INTRODUCTION

The term "sparganum" is used to describe the plerocercoid larva of pseudophyllidean cestodes of the genera Diphyllobothrium and Spirometra (Smyth and Heath, 1970). Infections due to these larval parasites are common and widespread, and earlier workers described them as a separate group of cestodes. This gave rise to the use of the name Sparganum in a generic sense whenever the specific identity of the adult was not known; this usage persisted even after it was evident that the term was an artificial one. More recently, the term has become a vernacular one.

An astonishing number of "species" of spargana have been described. This has led to an extremely complicated problem in classification, yet to be satisfactorily resolved (see below). The wide geographical distribution of spargana and the large range of animals which can act as intermediate hosts for these parasites make them important parasites for study. Human infection by spargana, known as human sparganosis, also has a wide geographical distribution, although it is especially serious as a public health problem in Asian countries.

This chapter is not a detailed review of literature since several excellent reviews have already been written on the subject (Huang and Kirk, 1962; Mueller, 1966; Smyth and Heath, 1970). This account merely serves as an outline of sparganosis and as an introduction to the following study of the sparganum of Spirometra erinacei.

Classification

Diesing 1854, originally proposed that the term Sparganum be used "as a generic name for any unidentified pseudophyllidean larva" (Wardle and McLeod, 1952). This usage is now considered artificial (Smyth and Heath, 1970).

The classification of this group of parasites is, however, still extremely complicated and unresolved. Vik (1964) has pointed out that the classification of even the genera Diphyllobothrium and Spirometra is still largely uncertain since "the validity of the generic as well as the specific features is still an unsolved problem." In two recent comprehensive works on cestodes (Wardle and McLeod, 1952; Yamaguti, 1959) the striking differences in the classification of the family Diphyllobothriidae by the authors give a good indication of the difficulties of classification confronting workers. Originally the family was called Dibothriocephalidae

Luhe, 1902. According to Wardle and McLeod (1952), Luhe (1910) changed the name to Diphyllobothriidae when he accepted that the tapeworm he named Dibothriocephalus latus belonged to the same genus as Cobbold's (1858) Diphyllobothrium stemmacephalum; Luhe (1910) thus renamed his tapeworm Diphyllobothrium latum.

Wardle and McLeod (1952) did not accept this change and retained the family name Dibothriocephalidae Luhe, 1902, and named 17 genera within that family. Yamaguti (1959), however, accepted the change and described the family Diphyllobothriidae Luhe, 1910, which contained 8 genera.

Both Wardle and McLeod (1952) and Yamaguti (1959) accepted the genus Spirometra, which was first proposed by Mueller (1937). That Spirometra and Diphyllobothrium are distinct genera was also accepted by Vik (1964) on morphological and biological grounds.

Wardle and McLeod (1952) named a total of 17 species within the genus Spirometra, including S. erinacei (Rudolphi, 1819), the species used in the present study (see Chapter 2). S. erinacei was also accepted as a valid species by Yamaguti (1959). However, it should be noted that the question of the status of the numerous species within the genus is extremely uncertain and

controversial (Wardle and McLeod, 1952).

S. erinacei has been described in a number of intermediate hosts in Australia (Bearup, 1948, 1953; Sandars, 1953, 1954; Gordon, Forsyth and Robinson, 1954) and the life cycle has been studied by Bearup (1953). Bearup (1948, 1953) used Iwata's (1933) description of S. erinacei for identification of the species in Australia, and the same authority has been followed for the identification of S. erinacei in the present study. Iwata (1933) synonymised most species described up to that time with S. erinacei and it would appear that this was a valid decision.

Life cycle

For a detailed review of the life cycle of sparganum-type cestodes, see Huang and Kirk (1962). A brief outline of the life cycle is as follows: --

Eggs are liberated in large number from the distal segments of the adult worm as they are passed out in the faeces of the definitive host. These eggs hatch in water and a free-swimming ciliated larva, the coracidium, is released. On ingestion by copepods, the coracidium penetrates the gut and reaches the haemocoel where it

develops into the next stage known as the procercoid. When the first intermediate host is ingested by a second intermediate host e.g. a frog, the procercoid penetrates the gut and develops into a plerocercoid, usually in an intramuscular site, within this host. The plerocercoid larva is known as the sparganum; if it is ingested by a suitable host e.g. a dog, it attaches itself to the intestinal wall and matures into the typically segmented adult. If, however, the sparganum is ingested by another intermediate host it will penetrate through the intestine and reestablish itself as a plerocercoid in the muscles and other tissue. In this way it can pass from one intermediate host to another as long as a definitive host does not intervene. The sparganum can infect a wide range of intermediate hosts including several species of amphibians, reptiles, birds and mammals (Huang and Kirk, 1962).

According to Huang and Kirk (1962) a number of workers have failed to establish spargana in frogs by feeding them copepods infected with procercoids, and passage of procercoids through tadpoles has been assumed by many to be an obligatory phase in the life cycle. The results of Bearup (1953) on S. erinacei would seem to lend support

to this view. He found that he could not infect pigs and rats by feeding procercoids, and only one of 12 mice became infected following this procedure. However, spargana readily became reestablished in all these hosts, as well as frogs, following ingestion of tadpoles infected by ingesting copepods with procercoids.

On the other hand, Mueller (1938a) has been able to infect mice with spargana of S. mansonoides by feeding procercoids from infected copepods. Furthermore, Mueller (1938b) could also infect monkeys with S. mansonoides by the same route.

Thus it would appear that there are species differences with regard to the necessity for passage through tadpoles before spargana are infective to other intermediate hosts.

For a comprehensive review of the natural hosts of spargana in Australia, see Sandars (1953). Mueller (1966) has given a detailed review of the biology of the North American species, S. mansonoides.

Sparganosis

Although a number of species have been implicated in causing human sparganosis (Smyth and Heath, 1970), in most cases, the actual species involved has not been established.

The geographical distribution of human sparganosis is extremely wide. Faust and Russell (1964) listed China, Japan, Formosa, Korea, and Vietnam as countries with high incidence of sparganosis, with a lower incidence in Africa, Australia, Indonesia, Holland, British Guiana, Puerto Rico, Honduras, Uruguay, Colombia and 12 states in the U.S. Huang and Kirk (1962) also listed Italy besides the ones mentioned, and Belding (1965) added Malaya to the list. Reviews on the pathology of sparganosis have been published by Huang and Kirk (1962) and Smyth and Heath (1970); a discussion on the epidemiology of sparganosis in Australia is given by Sandars (1954).

CHAPTER 2

IDENTIFICATION OF THE SPARGANUM OF *SPIROMETRA ERINACEI* AND ITS PENETRATION INTO THE INTERMEDIATE HOST

INTRODUCTION

The sparganum or plerocercoid of species of the genus *Spirometra*, represents the terminal larval stage in the life history of the parasite. If it is ingested by a host in which it cannot mature into an adult, the sparganum will often penetrate the gut and reestablish itself in another part of the body. It appears that provided a definitive host does not intervene, spargana may be passaged successively through intermediate hosts which may follow one another in a particular food chain.

The present chapter reports studies made to determine the identity of spargana recovered from black snakes and Queensland cane toads, and to investigate penetration of the gut of the mouse which was found to act as a satisfactory laboratory intermediate host for the parasite.

METHODS AND MATERIALS

(a) Identification of the sparganum

Spargana were collected from two different hosts: Queensland cane toads, Bufo marinus, from Innisfail, Queensland, and the black snake, Demansia textilis textilis, from the Lake George area, N.S.W. It was necessary to establish the species of sparganum from cane toads since these animals have not been previously recorded as hosts of spargana in Australia (see Sandars, 1953). Accordingly, two dogs were fed with spargana to obtain adult worms for identification purposes.

The first dog was fed 15 spargana collected from Bufo marinus. On the 10th day following infection, eggs began to appear in the faeces of the dog, and these were washed in distilled water and examined under the microscope. On the 23rd day several pieces of strobila consisting of gravid proglottids were found in the faeces. These were washed, stained with Fast Red Salt B (Johri and Smyth, 1956), dehydrated in alcohol and mounted in Canada balsam. Two days later the dog was dosed with Hydatex but no adult cestodes were found. This dog was not killed because it was required for other experiments.

A second dog was fed 2 spargana collected from the black snake Demansia textilis textilis, and 2 spargana from Bufo marinus. The dog was killed 28 days later and immediately dissected. About 2 feet of the small intestine immediately distal to the pylorus was rapidly removed, tied at both ends, and injected with warm Bouin's fixative. The entire piece of gut was then opened longitudinally and immersed in Bouin's fixative for 48 hrs. Four adult cestodes were found attached to the duodenum, about 15 cm from the pyloric sphincter and separated from each other by a few mm. No attempt was made to remove the scoleces of the worms as they were firmly attached. Instead, pieces of gut with the attached scoleces were excised, dehydrated and embedded in paraffin in the usual manner. Sections of the scolex were then cut at 6μ-8μ and stained with either haematoxylin and eosin or by the Maximow technique for morphological details.

Pieces of the strobila were also excised and some were stained with Fast Red Salt B (Johri and Smyth, 1956) and mounted in balsam. The remainder were embedded in paraffin in the usual manner and sections were cut at 6μ-8μ, and stained with haematoxylin and eosin for morphological studies.

(b) Penetration experiments

Nearly all the spargana of S. erinacei used for the experiments were obtained from Queensland cane toads, Bufo marinus, which were collected by the Apex Club of Innisfail, and sent to Canberra. On two occasions, some spargana were dissected from the black snake, Demansia textilis textilis collected from the Lake George area, N.S.W.

The spargana were found to be either free or encapsulated. The free specimens were found lying either just below the skin in the superficial connective tissues or entwined among the muscles. The encapsulated specimens were firmly enclosed in tough yellowish capsules which varied from 3 mm to 10 mm in diameter (see Fig. 4A). The free spargana seemed to be much more vigorous and active than those released mechanically from their capsules.

Initially spargana were fed to laboratory mice (strain Quakenbush) in different forms, i.e. either intact capsules, or spargana dissected from capsules, or whole non-encapsulated spargana. In later infections approximately 10 mm of the scolex and anterior end of free spargana were used. In all cases the mice were forced fed while lightly anaesthetised with ether.

FIGURE 1

T.S. of scolex of adult Spirometra erinacei in duodenum of dog.

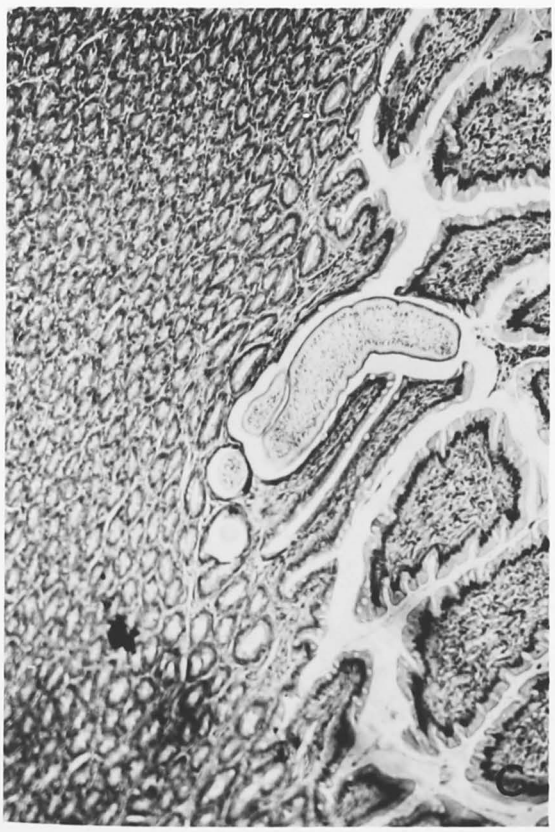
A - Bothria clamped around villi for attachment. (H&E) x50

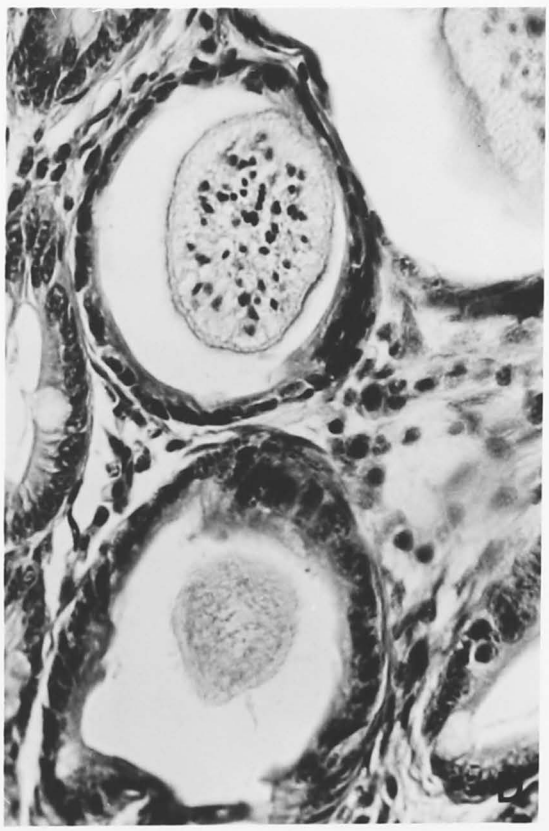
B - As above. (PAS) x50

C - Tip of scolex in intestinal crypts. (H&E) x50

D - Tip of scolex in crypts. (H&E) x350


A





A series of mice were fed one to three spargana each and killed after 10 min, 20 min, 30 min, 40 min, 50 min, 1 hr, 24 hr, 48 hr, 72 hr, and 2 mth, respectively. They were dissected rapidly after being killed and the locations of spargana were recorded. These infections were carried out on different occasions due to the unavailability of large numbers of spargana at any one time. Some of the infections were, however, repeated several times. When it was found necessary to hold some spargana until sufficient had been collected for experiments, the spargana were washed once in Ringer's solution after removal from the host and maintained in 20 ml of Ringer's in closed Petri dishes at 4°C. The spargana were never used later than 48 hr after removal from the host.

RESULTS

(a) Identification of the sparganum

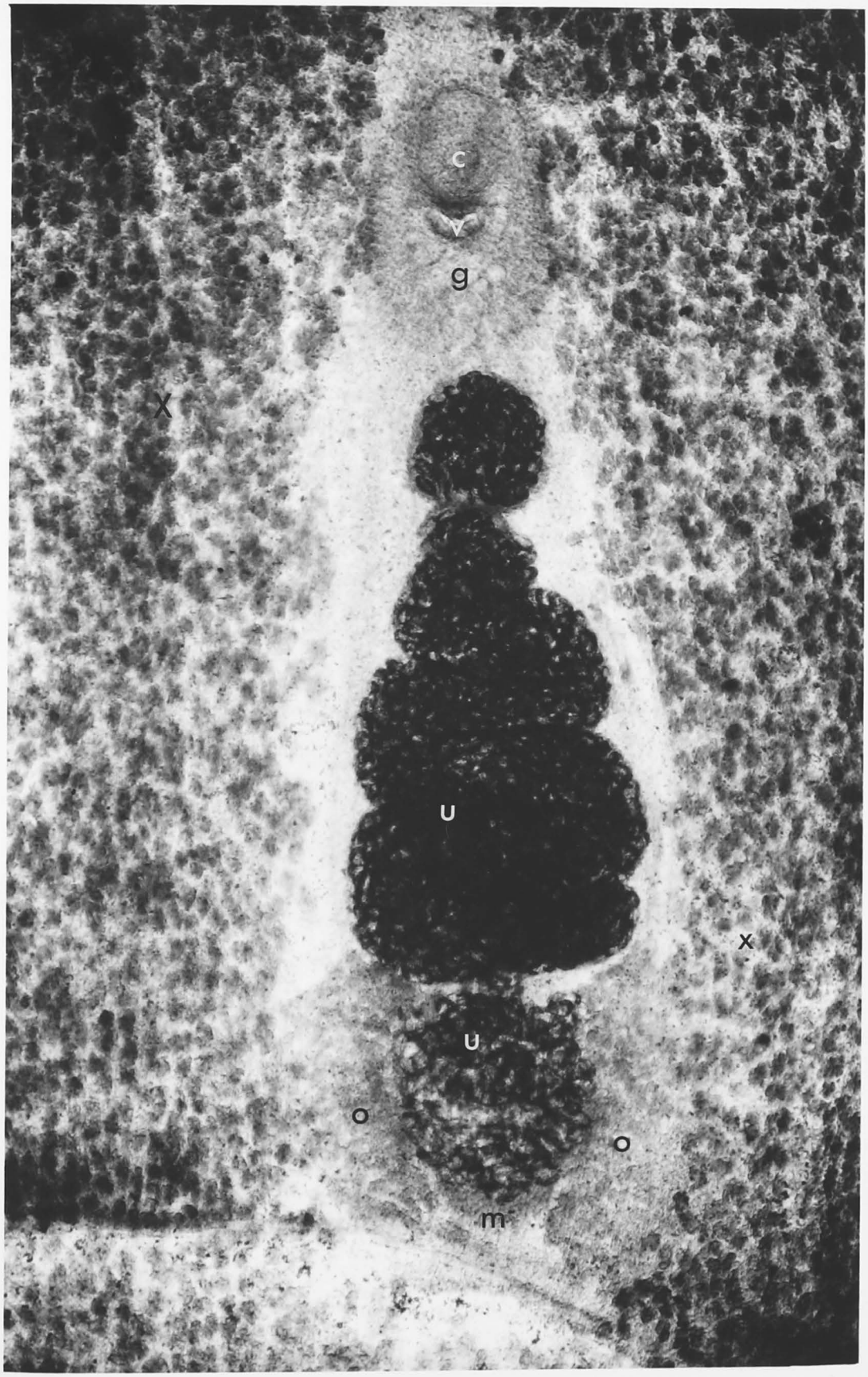
In the first dog infected, only gravid proglottids and eggs were recovered. The adult(s), presumably derived from spargana of cane toad origin, were not recovered following dosing.

In the second dog infected, 4 sexually mature adults

FIGURE 2

Proglottid of adult S. erinacei. (Fast Red) x50

List of abbreviations:
c-cirrus pore; v-vaginal pore; g-genital pouch; u-uterus; o-ovary; m-Mehlis' gland; x-testes and vitellaria.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developed from the 4 spargana fed -- 2 each from the cane toad and black snake respectively. Although in this case it was impossible to establish the intermediate host origin of the respective adults, there did not appear to be any major differences between them with regard to their morphology and accordingly, all 4 are regarded here as the same species. The morphology of gravid material recovered from the first dog agreed with material from second dog.

The following description is based on the material recovered from the two dogs infected. The colour of the adult is grayish or milky white. The scolex lacks suckers and hooks but bears shallow bothria on the dorsal and ventral surfaces. In cross-section the scolex has a shape as seen in Fig. 1,A, and is approximately 0.5 mm wide.

Sections of the adult scolex illustrate the mode of attachment of adult S. erinacei in the intestine of the dog (see Fig. 1,A and B). The bothria are firmly clamped around villi; the tip of the scolex may lie between villi, but sometimes penetrates into the crypts of Lieberkühn (see Fig. 1,C and D). However, no pronounced distension of the crypts was observed and very little

tissue damage was evident. Although the epithelial cells of the villi sometimes appear to be firmly compressed by the bothria, no necrosis, or destruction of cell membranes was apparent. The tegument of the adult scolex is AB positive (Fig. 3,B) but PAS negative (Fig. 1,B) which is the converse of the situation in the sparganum (see Chapter 3).

In the following description, the terminology of Iwata (1933) is followed.

The total length of the strobila is about 50 cm long, about 8 mm wide and 1 mm thick. The mature proglottids are usually near the last quarter of the strobila, but this is variable since groups of gravid proglottids are constantly shed by the worm. The shape of the proglottids is also variable; the more anterior ones are usually broader than long, whereas the most posterior ones are longer than broad (see Fig. 2).

The testes are found in the medullary layer and are oval or lobed in shape, about 110μ x 160μ in size (Fig. 3,A). The testes (as well as the vitellaria) are absent from the central regions of the proglottid so that a clear area surrounds the genital pouch, uterus and ovary. In anterior proglottids, this clear area extends along the

FIGURE 3

A - T.S. of adult S. erinacei proglottid. (H&E) x100 V-vitellaria; T-testes.

B - T.S. of adult scolex. (AB) x100

C - L.S. of sparganum scolex, protruded. (H&E) x30

D - L.S. of sparganum scolex, invaginated. (Azan) x30


V
T
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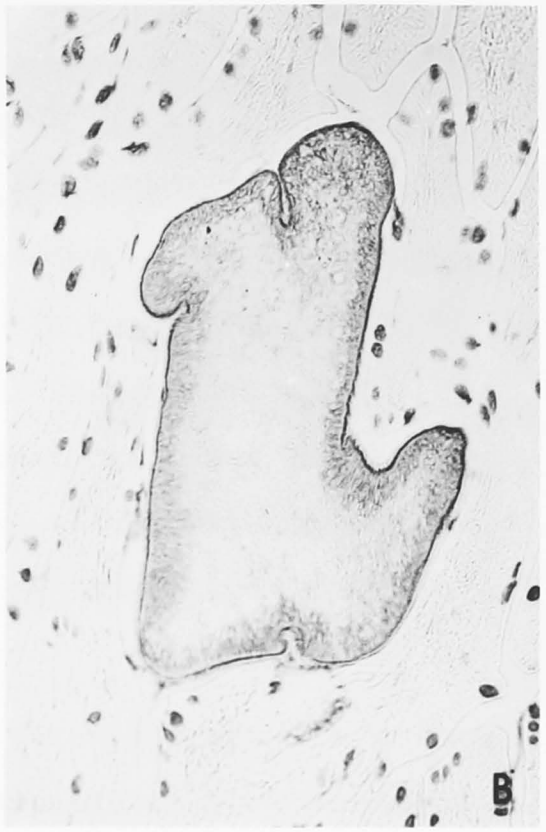
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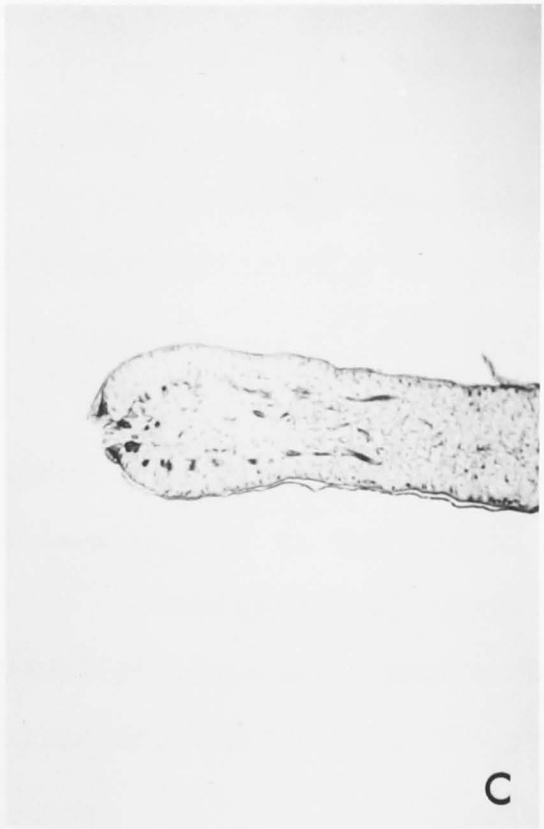
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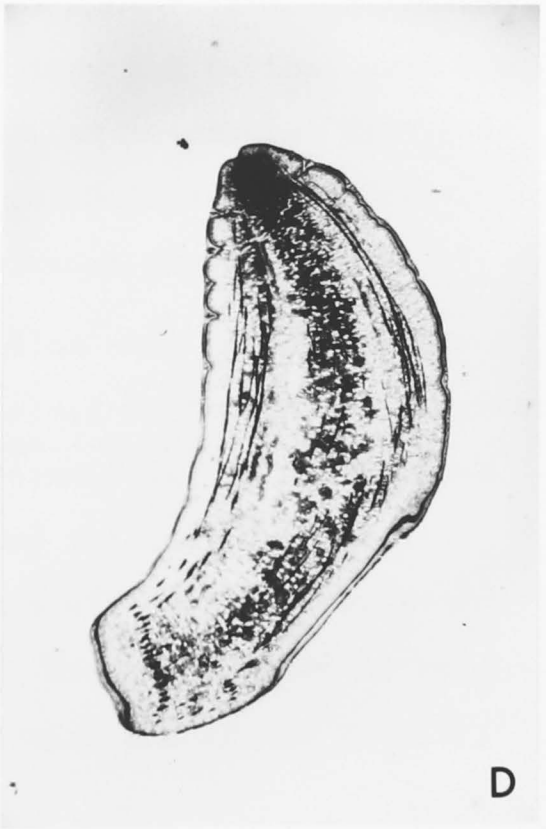
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entire axis so that the testes are confined to the lateral regions of the proglottid. In the longer posterior proglottids, however, the testes are situated laterally as well as across the anterior end of the proglottid, meeting in front of the genital pouch. The cirrus sac is oval in shape and is situated below the genital pouch. It communicates to the exterior via a cirrus pore which is situated at the apex of the genital pouch.

Three ventrally situated pores are present in each mature proglottid; these are the cirrus pore and vaginal pore, which are located more anteriorly than the uterine pore (see Fig. 2). The cirrus pore and vaginal pore are united to form the genital pouch in the median axis. In long proglottids, the genital pouch is situated some distance anteriorly from the uterine pore.

The uterus lies in the median axis and extends from the posterior end of the proglottid as far as the anterior third. It is spiral in form, consisting of about three to six coils; in the vicinity of the uterine pore it forms a hemispherical mass. The width of the coils usually increases posteriorly. The ovary tends to be dendritic and is near the posterior end of the proglottid;

it lies ventral to and on either side of the proximal portion of the uterus. The vitellaria are in the cortical layer of the proglottid and each follicle is oval in shape. The vitellaria sometimes obscure the ovary in long proglottids. The eggs measure about 57 μ to 66 μ x 32 μ to 36 μ. They are oval in shape but tend to be slightly assymetrical with one side rounder than the other; both ends are slightly pointed. An operculum is present at the sharper end of the egg.

The above description agrees substantially with that given by Iwata (1933) for D. erinacei. Significant and consistent features which characterise this species are egg size, egg shape, structure of the scolex with bothria on dorsal and ventral sides, reproductive organs one pair in each proglottid, uterus forming spiral coils, and the uterine pore and cirrus pore opening on the ventral side in the median axis. D. erinacei, however, has been transferred to the genus Spirometra (Wardle and McLeod, 1952) and this change in generic status has been accepted by later workers (Yamaguti, 1959).

Thus, the spargana from Bufo marinus, the Queensland cane toad from Innisfail, Queensland, and Demansia textilis textilis, the black snake from Lake George, N.S.W., can

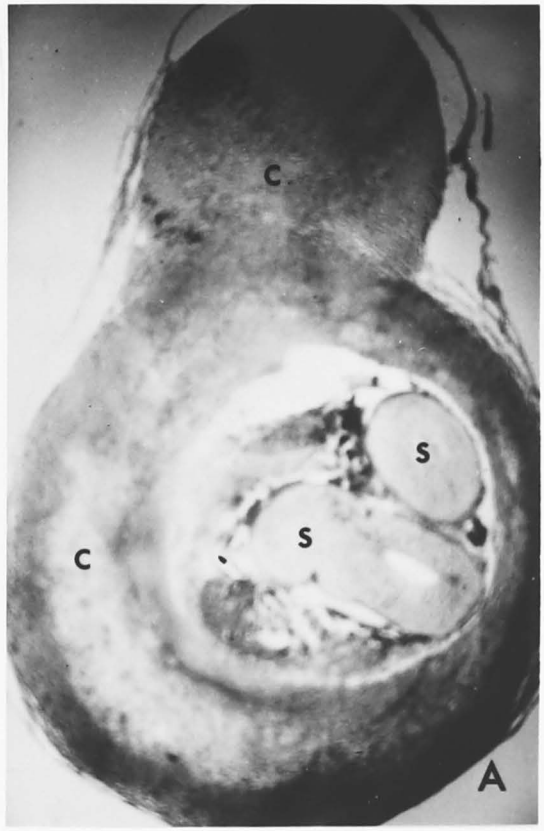
FIGURE 4

A - Sparganum in host capsule of toad. (H&E) x20 s-sparganum; c-host capsule.

B - Sparganum penetrating duodenum of mouse. Note that damage to gut tissue is localised and minor. (H&E) x10

C - As in B. Note that villi not in immediate vicinity of perforation remain intact. (H&E) x30

D - As in B&C. Note absence of extensive damage to surrounding tissue. Arrow indicates debris, mainly nuclei of host mucosal cells. x50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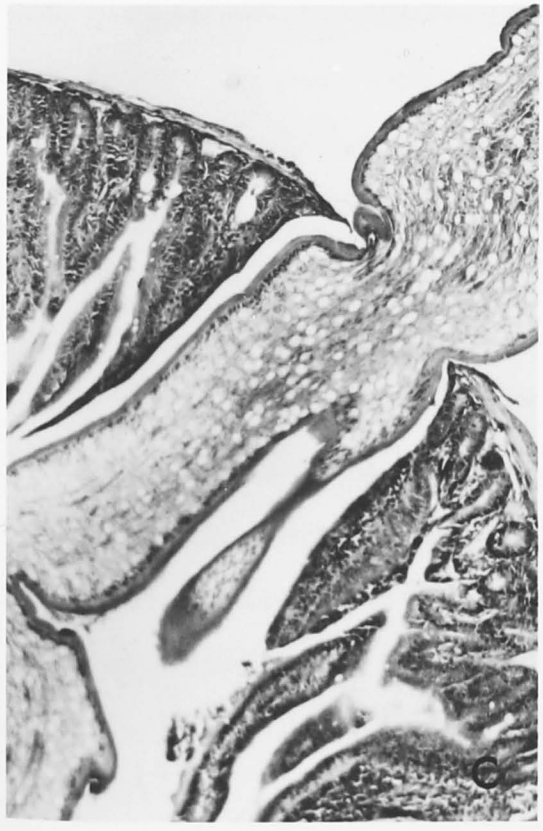
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D

be confidently referred to as S. erinacei, as characterised by Iwata (1933). S. erinacei spargana have been recovered from a variety of Australian host species previously (Bearup, 1948, 1953; Sandars, 1953; Gordon, Forsyth and Robinson, 1954).

(b) Penetration experiments

Mice fed with intact capsules were dissected 24 hr later but no spargana were found.

Both free spargana and those liberated mechanically from the capsules readily penetrated the gut of the mouse after oral administration. The point of penetration was always in the duodenum, about 2-4 cm below the pyloric sphincter. In one mouse, 3 spargana penetrated the duodenum within 1 mm of each other at different points around the intestinal wall. As shown in Fig. 5, penetration of the gut commences approximately 30 min after ingestion. This time lapse between ingestion and penetration was reasonably consistent on the 7 occasions this experiment was carried out. The actual process of gut penetration was observed to take only 5-10 min on most occasions, if no great disarrangement of the gut and its associated organs was made. If the duodenum was

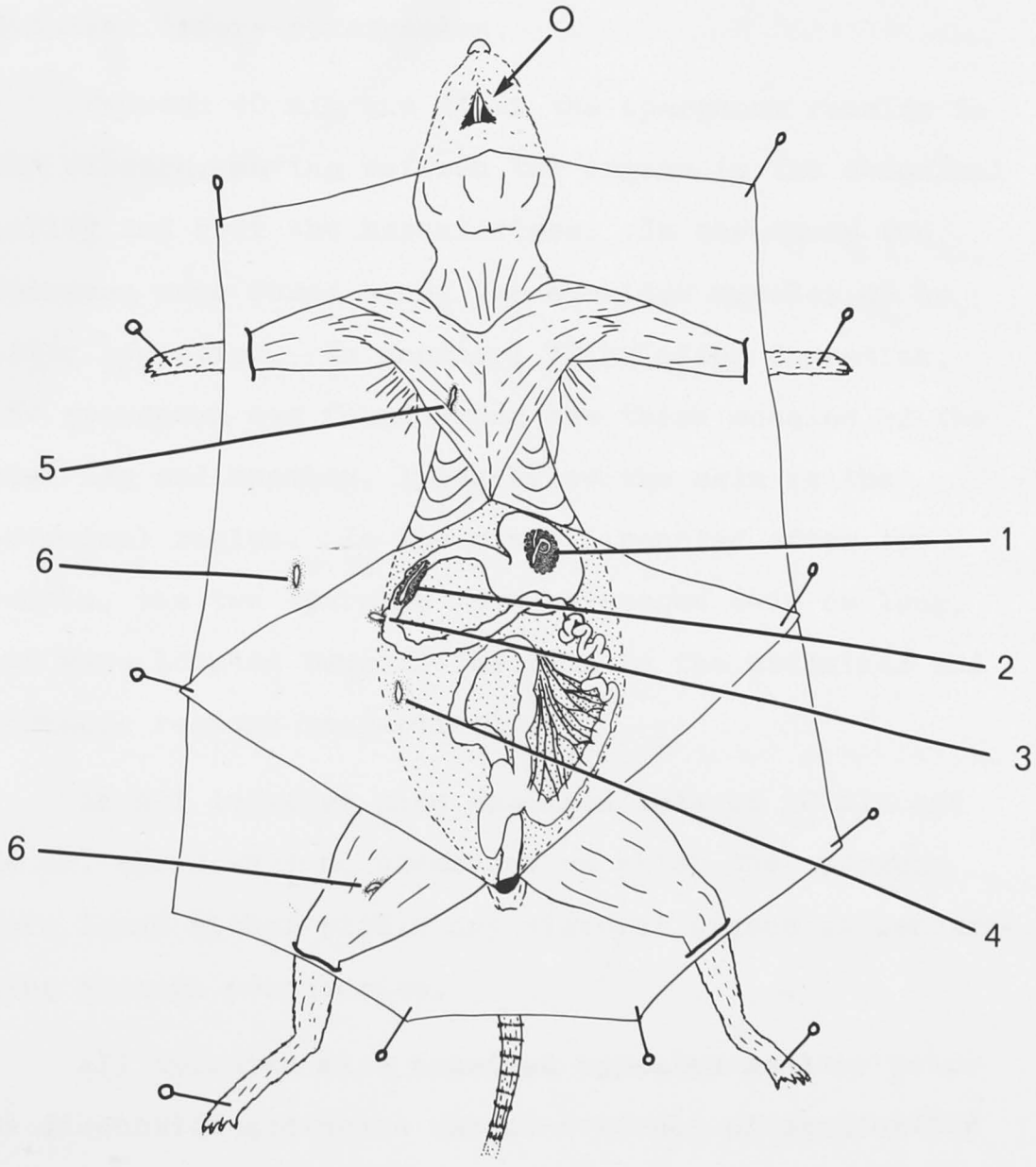
freed from remaining viscera to facilitate observation, penetration was delayed by up to 15 min. Only the scolex (2-5 mm of the anterior portion of the sparganum) emerged into the abdominal cavity from the gut. The rest of the strobila became detached posterior to the scolex and remained in the intestinal lumen. Fig. 4,B, shows a section through the penetrating sparganum. The size of the actual perforation caused by the penetrating sparganum is about 0.5 mm in diameter and constitutes a direct connection between the gut lumen and the coelom. In spite of the relatively large size of the perforation, however, damage to gut tissues appeared minor and localised, suggesting that the mechanism of penetration was direct and efficient (see Fig. 4,C and D). It is evident that the scolex breaks through the peritoneal lining of the gut into the abdominal cavity and does not remain attached to the gut just below the peritoneal lining.

Ten minutes after feeding, one sparganum was found in the stomach with the strobila still attached to the scolex. After 20 min a sparganum was found in the duodenum with the strobila already sloughed off, but at this stage the scolex had not penetrated the duodenum.

FIGURE 5

Sites of the sparganum in the mouse at various times following ingestion.

0. - Ingestion of sparganum by force-feeding.
1. - In the stomach, sparganum intact. 10 min after ingestion.
2. - In duodenum, scolex detached. 20 min
3. - Penetrating scolex. 30 min
4. - Scolex in the body cavity. 40 min - 24 hr
5. - Scolex among shoulder muscles. 48 hr
6. - Scolex among thigh muscles and connective tissue, immediately beneath skin. 72 hr - 2 months.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Hence, it appears the bulk of the strobila becomes detached before penetration.

Between 40 min and 24 hr the sparganum remains in the viscera, moving between the organs in the abdominal cavity and over the mesenteries. In one mouse two spargana were found among the shoulder muscles 48 hr after ingestion. In another, 72 hr after ingestion, one sparganum was found among the thigh muscles of the hind leg and another, lying below the skin in the abdominal region. In the mouse dissected after two months, the two spargana found averaged 8-10 cm long, and were located beneath the skin in the abdominal and thoracic regions respectively.

In all infected mice examined between 40 min and 24 hr, there were no occasions on which the spargana were found either within any visceral organs or penetrating through mesenteries.

All infected mice examined appeared healthy prior to dissection and there was no evidence of peritonitis which might be considered possible as a result of the fairly large perforation made by the sparganum in the gut wall. No inflammation at or around the site of penetration was evident, nor did there appear to be any

significant host reaction to spargana in tissue sites. However, the latter point was not checked histologically.

DISCUSSION

The identification of the spargana from Bufo marinus (Innisfail, Qld.) as belonging to the species Spirometra erinacei (Rudolphi, 1819) has established a new host for this parasite. Sandars (1953) has already noted spargana of this species in Demansia textilis in Australia.

From the results of penetration experiments described above, the sparganum of S. erinacei was shown to complete penetration of the duodenum of a mouse within 40 min after ingestion. The actual time taken for penetration was, however, much less than this since 10 min after ingestion the sparganum was still present in the stomach, and even after 20 min penetration had not commenced. It was, in fact, observed that penetration commenced only after 30 min and the entire process of gut penetration took only 5 to 10 min to complete. The times recorded for the penetration of mouse intestine by S. erinacei are considerably less than those recorded for the spargana of S. mansonoides by Mueller (1938b). Mueller found that the sparganum of S. mansonoides penetrated mouse gut

1 to 1¼ hr after ingestion and penetration itself took 20 to 30 min to complete. The results of Takahashi (1959a), which showed that the sparganum of D. mansoni (= S. erinacei ?) penetrated the mouse gut within 40 min after ingestion, is however consistent with the observations of the present study.

Any significant disarrangement of the gut during penetration was seen to delay penetration by S. erinacei spargana by up to 15 min. This occurred in cases when the duodenal loop was pulled away from the rest of the viscera to facilitate observation. When this was done before penetration had begun, the sparganum failed to penetrate the gut or even initiate penetration. Similarly, a sparganum failed to penetrate when a section of the duodenum with an enclosed sparganum was removed, tied at both ends and immersed in Ringer's solution at 37°C. These results were not surprising, since a great deal of physiological damage was probably done to the gut as a result of such drastic treatment, which probably interfered with sparganum penetration.

What is difficult to understand is why peritonitis did not occur in mice following infection with spargana. The perforation in the gut wall made by the penetrating

sparganum is about 0.5 mm in diameter and is thus a relatively large opening. Contents of the intestinal lumen (including bacteria and other organisms) would presumably be released into the peritoneal cavity as the sparganum penetrates. These views receive support from studies by Feng and Hoeppli (1936) with spargana of S. erinacei in Chinese hamsters. In this host, the spargana perforated the gut wall and thus establish a connection between the intestinal lumen and peritoneal cavity. Feng and Hoeppli found that bacteria and other organisms were released into the peritoneal cavity through the perforation. Moreover, they established beyond doubt that the absence of fatal peritonitis was due to bacteriacidal properties possessed by the hamster. They repeated experiments earlier made by Joyeux and Baer (1929) and found no evidence that the spargana possessed bacteriacidal properties. However, Joyeux and Baer (1929) may have been dealing with a different species. It is not unlikely that mice may possess a similar mechanism to hamsters in preventing fatal peritonitis due to penetrating spargana, since a mouse infected with two spargana was still in an apparently healthy state after two months.

In contrast to the spargana of S. erinacei, the spargana of S. mansonoides, after penetrating through the mucosa of the mouse intestine, do not pass directly into the body cavity but migrated tangentially beneath the serosa and finally escaped where the mesentery joins the gut (Mueller, 1938b). Sometimes the sparganum of S. mansonoides travelled upwards between the two coats of the mesentery and reach the muscles via that route. Thus Mueller (1938b) explained that no infection can occur in the mouse since "no direct communication is formed between the cavity of the intestine and the outside and hence the infective contents of the gut do not escape into the coelom".

Another feature of the penetrating sparganum of S. erinacei noted in this study was that only about 2-5 mm of the scolex reached the peritoneal cavity. The major part of the strobila was discarded before penetration and remained in the duodenum of the mouse. It appears possible that one of the factors which activate the sparganum to detach its scolex from the rest of the larval body is an increase in temperature to 37°C. When a sparganum kept in Ringer's solution at room temperature is placed into a dish of Ringer's at 37°C, the activity

of the sparganum is significantly increased and within 15 to 25 min the scolex separates from the remainder of the strobila. This time interval coincides with that in which an ingested sparganum is found in the duodenum of the host with the scolex detached from the strobila.

CHAPTER 3

THE MORPHOLOGY AND HISTOCHEMISTRY OF THE SPARGANUM SCOLEX OF SPIROMETRA ERINACEI

INTRODUCTION

The sparganum of S. erinacei is able to rapidly penetrate the gut of an intermediate host following ingestion and become established as a tissue parasite (see Chapter 2). A number of studies (Li, 1929; Mueller, 1938a, 1938b; Bearup, 1953; Sandars, 1953; Takahashi, 1959a) of the spargana of S. erinacei and related species have been made but the mechanism of penetration has not yet been established. The remarkable migration of the sparganum is accomplished despite the fact that it lacks any striking modifications of the scolex which might facilitate mechanical penetration through the relatively thick muscular and collagenous layers of the host intestine. Thus, it would seem that any mechanical effects exerted by the scolex might have to be augmented by some other mechanism to effect penetration.

Smyth and Heath (1970) have suggested that "cephalic glands" are present in spargana and that these may be histolytic in nature and possibly involved in the process

of penetration. However, evidence for the existence of such glands in Spirometrid pseudophyllidean cestodes is lacking. Glands have been described in the plerocercoid scolex of several other species of pseudophyllidean cestodes, including Diphyllobothrium latum, D. vogeli, D. osmeri, D. dendriticum (Kuhlow, 1953) D. dalliae, D. norvegicum (Vik, 1964), as well as in the adult scolex of Dibothriocephalus wilsoni, Adenocephalus pacificus and Glandicephalus antarticus (Wardle and McLeod, 1952), and Abothrium gadi (Williams, 1960). "Histolytic glands" have also been described in the procercoid of Spirometra erinacei by Li (1929).

A study of the morphology and histochemistry of the scolex of the sparganum of S. erinacei, at the light microscope level, was therefore undertaken initially to establish whether recognisable gland cells are present in this region which might be linked with penetration. This chapter describes the results of this investigation.

METHODS AND MATERIALS

(a) Morphological study of the sparganum scolex

Spargana removed from B. marinus and D. textilis textilis were placed in Petri dishes of Ringer's solution

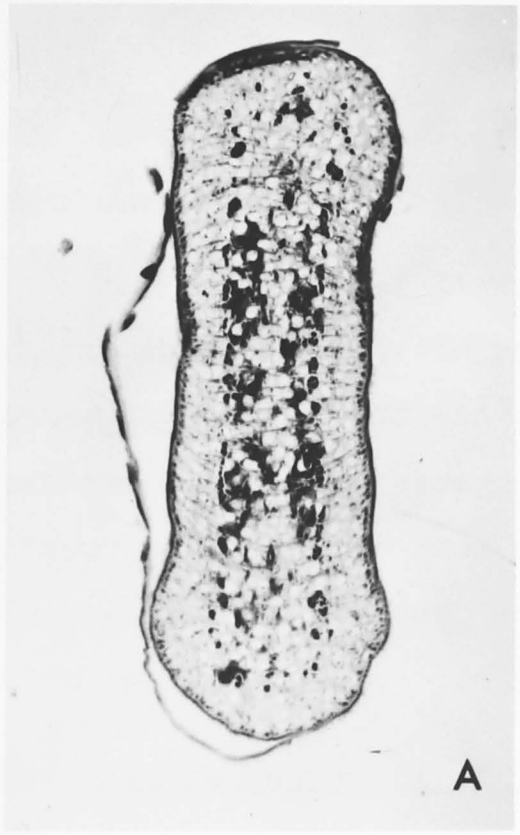
FIGURE 6

A - T.S. of sparganum scolex. (HgBPB) x100

B - As above. x300

C - L.S. of sparganum scolex. (Azan) x300

D - As in C. Note that cytoplasmic extensions of the tegumental cells are visible. x500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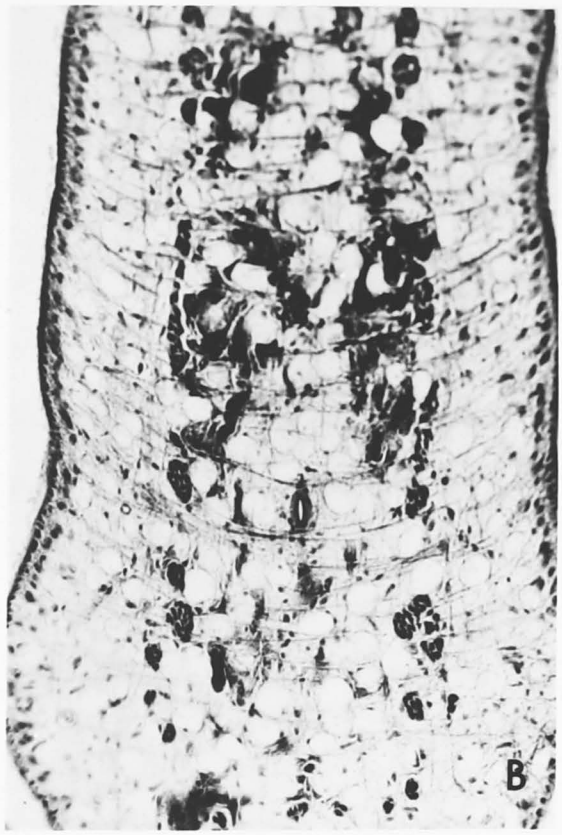
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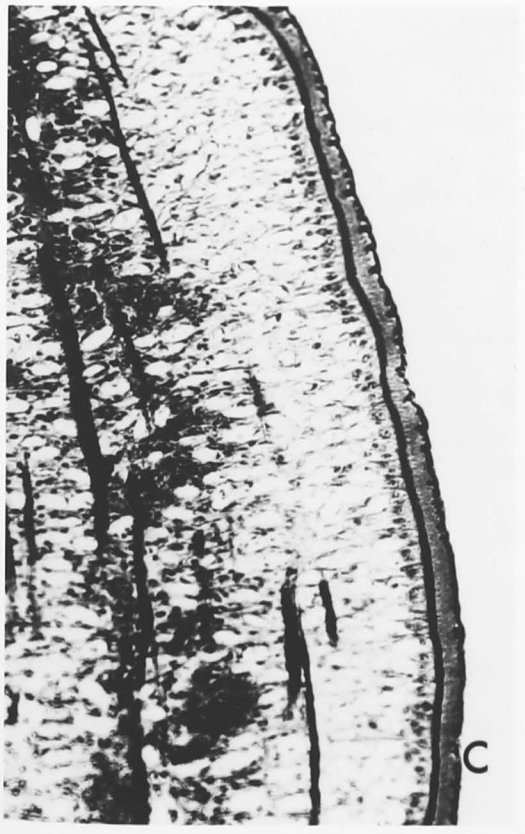
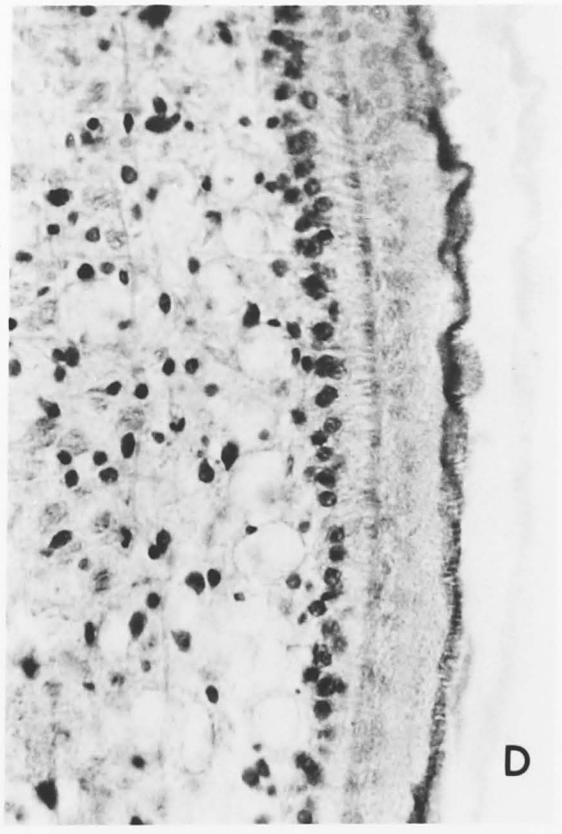

C


D

(B.D.H. tablets) and studied under a low power binocular microscope.

For the study of living worms at higher magnifications, about 2 cm of the anterior portion of the sparganum was cut off and flattened with gentle pressure between a slide and cover slip. Care was taken to prevent preparations from drying out during examination. Neutral Red (1% aqueous) was used on occasions as a vital stain.

(b) Histological study of the sparganum scolex

The spargana used for histological studies were either collected directly from the natural host (e.g. B. marinus), or were first fed to laboratory mice (Quakenbush strain) and then collected after penetration of the gut (see Chapter 2).

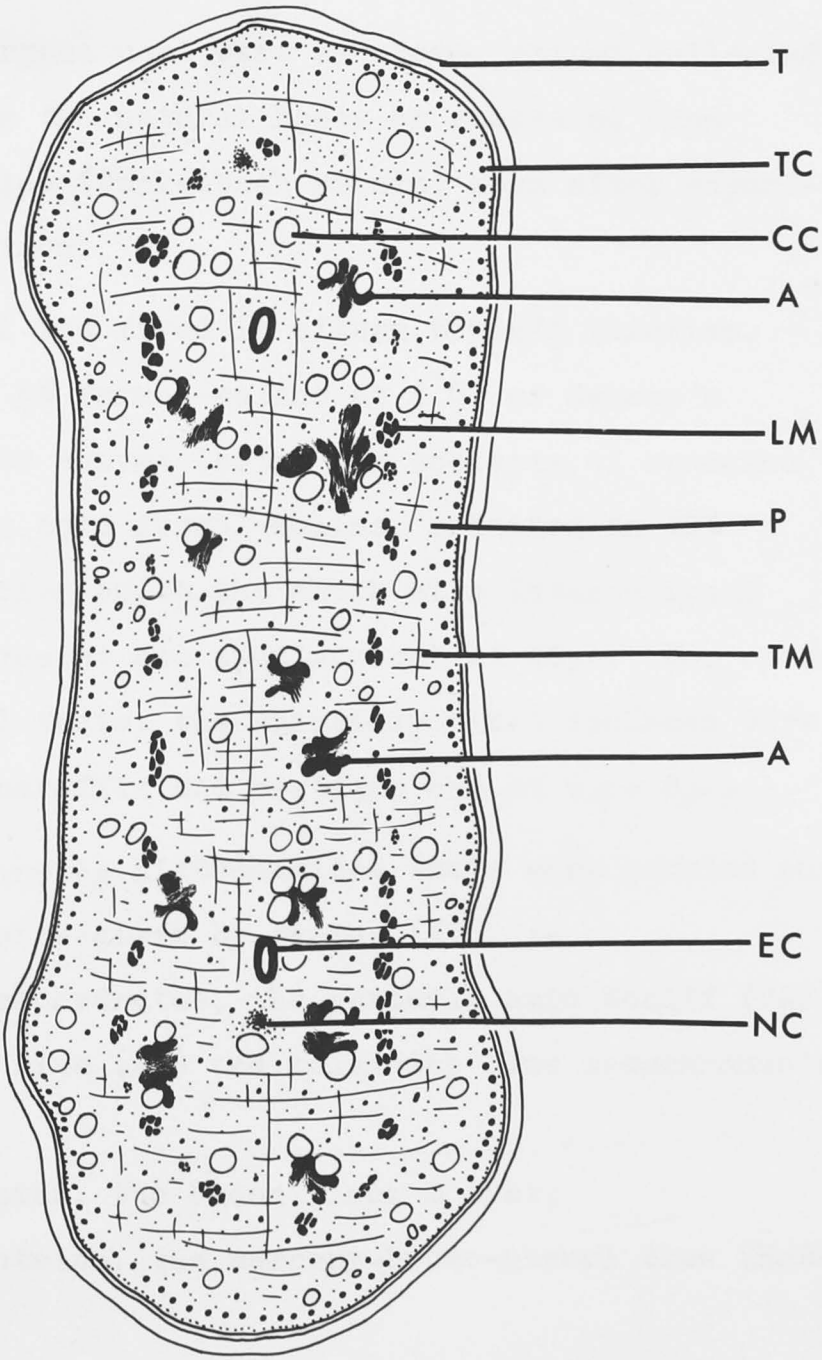
The spargana were washed in Ringer's and 3-5 mm of the scolex was cut off. They were then fixed in Bouin's and Carnoy's fixatives overnight, dehydrated in alcohol, and embedded in paraffin in the usual manner. Sections were cut at 6μ- 8$\mu$and stained with haematoxylin and eosin. Some sections were stained with Heidenhain's Azan technique (Conn, Darrow and Emmel, 1960).

FIGURE 7

Transverse section through sparganum scolex, illustrating the distribution of structures. x100

List of abbreviations:

T-tegument; TC-tegumental cell; CC-calcareous corpuscle; A-amorphous body; LM-longitudinal muscles; P-parenchyma; TM-transverse muscles; EC-excretory canal; NC-nerve cord.


T
TC
CC
A
LM
P
TM
A
EC
NC

(c) Histochemical study of the sparganum scolex

The spargana used were as above, either collected directly from the natural hosts or recovered from laboratory mice (Quakenbush strain) soon after experimental infection.

Material was fixed in either Bouin's fixative, 70% alcohol, 4% formol-saline at 4°C, or Carnoy's fixative. For enzyme tests, the scoleces of spargana were fixed in cold formol-saline, embedded in 10% gelatine, frozen on to the chuck of a International model CTD cryostat and sections cut at 20μ. For other histochemical tests, the variously fixed scoleces were embedded in paraffin and sections cut at 6μ - 8μ.

The following histochemical tests were carried out using procedures given by Pearse (1961):-

(i) for carbohydrates, the periodic acid Schiff (PAS), alcian blue (AB) and toluidine blue metachromasia tests;

(ii) for lipids, the Sudan black B test;

(iii) for proteins, the mercury-bromo-phenol blue (HgBPB) test;

(iv) for RNA and DNA, the methyl green pyronin (MGP) test;

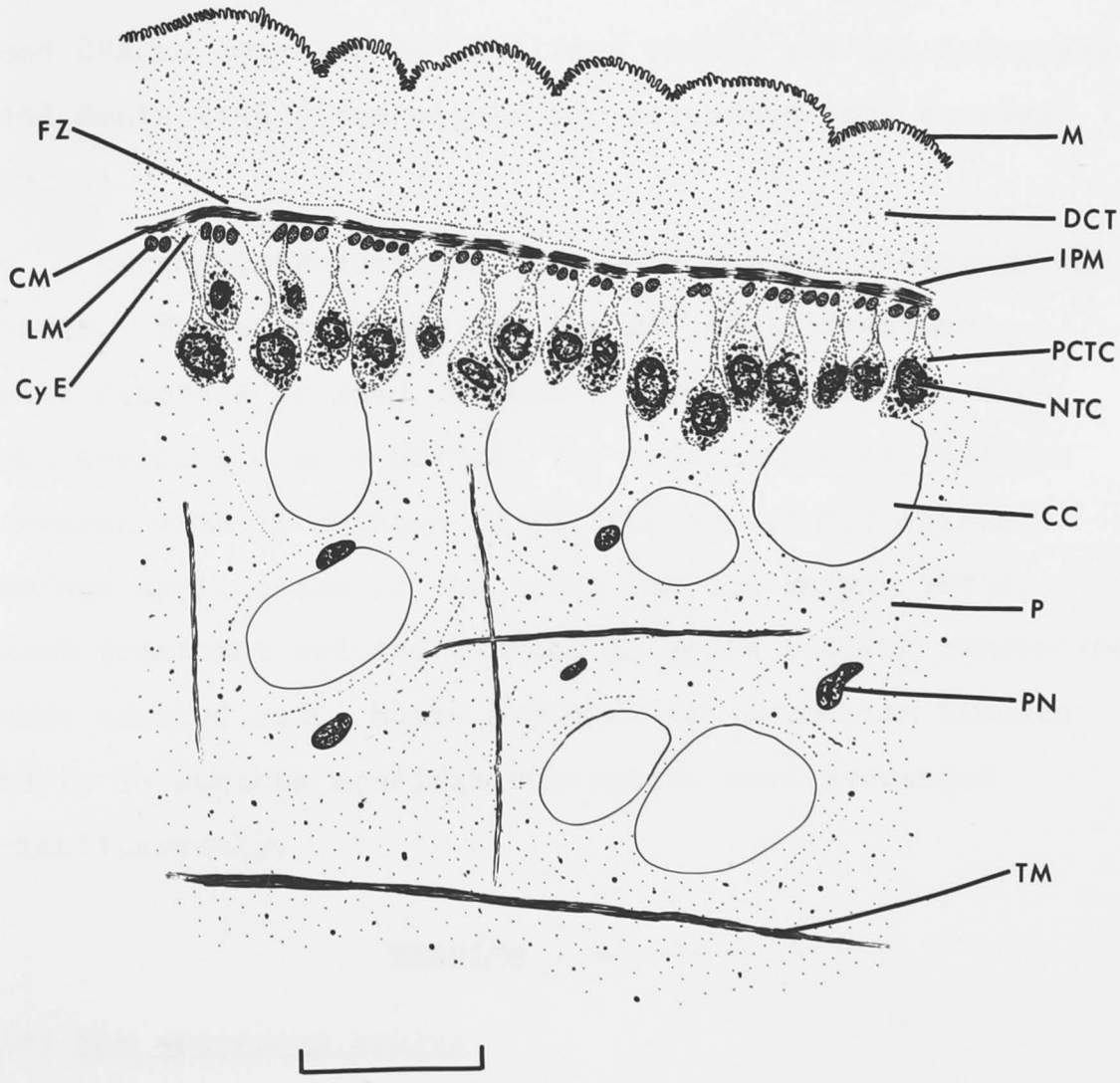
(v) for enzymes, the Gomori methods for acid and alkaline

FIGURE 8

Transverse section through sparganum scolex, in the tegumental region. (Scale line = 10μ)

List of abbreviations:

M-microtriches; DCT-distal cytoplasm of the tegument; IPM-internal plasma membrane; FZ-fibrous zone; CM-circular muscles; LM-longitudinal muscles; CyE-cytoplasmic extension; PCTC-perinuclear cytoplasm of the tegumental cell; NTC-nucleus of the tegumental cell; CC-calcareous corpuscle; P-parenchyma; PN-parenchymal nucleus; TM-transverse muscles.


FZ
M
DCT
CM
IPM
LM
PCTC
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NTC
CC
P
PN
TM

phosphatase, the Nachlas, Crawford and Seligman method for leucine amino-peptidase, the Holt and Withers method for esterase.

In addition, the acridine orange method for RNA and DNA as detailed by Culling(1963), and the Karnovsky and Roots (1964) techniques for esterases were carried out.

Appropriate controls were used for all histochemical tests. These included, for the PAS test, treatment with diastase (Sigma) to remove glycogen; for the MGP and acridine orange method, the examination of sections treated with 1% RNA-ase in PO_4 buffer, pH 7.3 (Sigma) before application of the test; for the enzyme tests, heat treatment and incubations in media lacking substrates were carried out. Where appropriate, mammalian tissues known to contain specific substances were processed simultaneously.

RESULTS

(a) The sparganum scolex

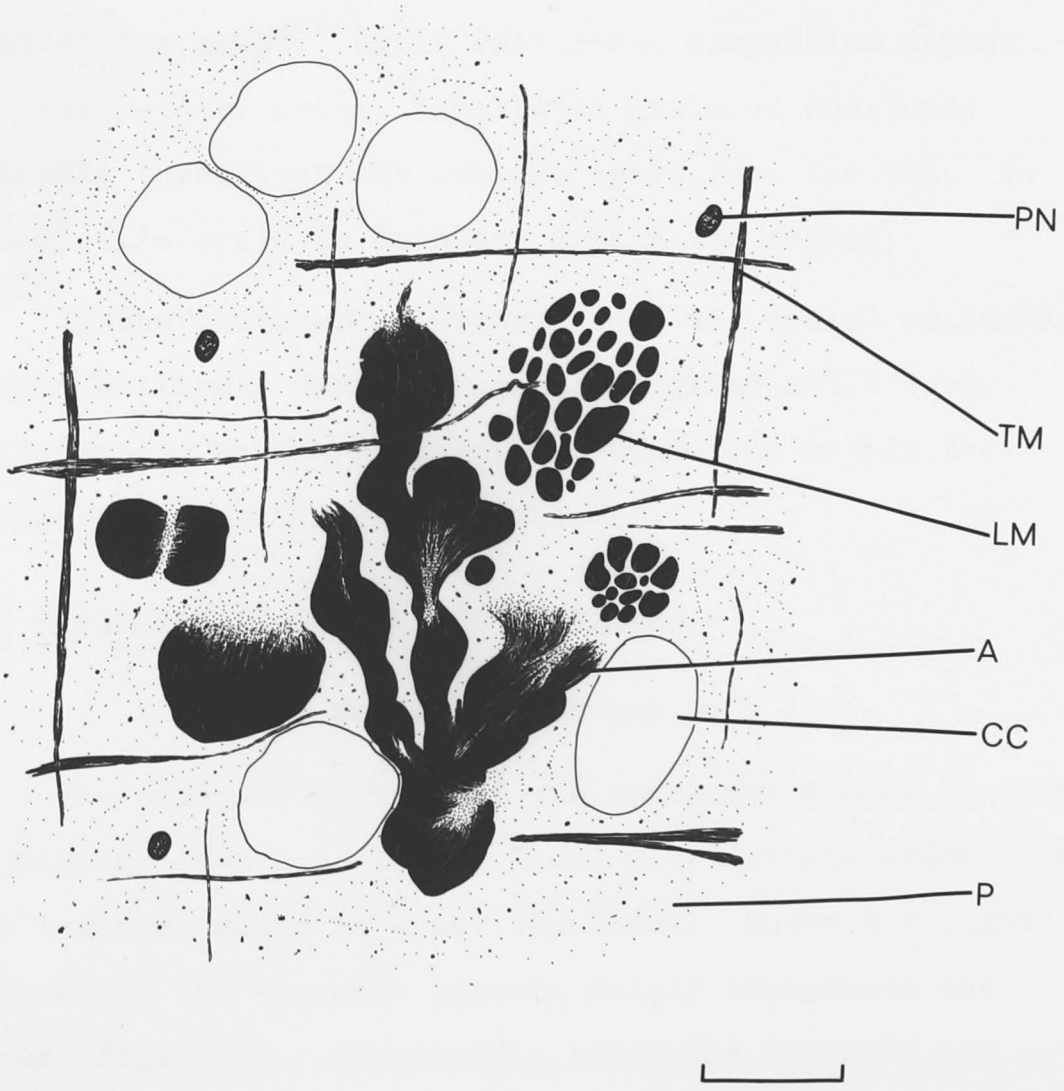
Spargana from D. textilis textilis measure up to 1 cm wide and 45 cm long, and are generally much larger than those from B. marinus (usually 2-5 mm wide and

FIGURE 9

Transverse section through sparganum scolex, in medullary region. (Scale line = 10μ)

List of abbreviations:

PN-parenchymal nucleus; TM-transverse muscles; LM-longitudinal muscles; A-amorphous body; CC-calcareous corpuscle; P-parenchyma.


PN
TM
LM
A
CC
P

8-10 cm long).

In live specimens, the scolex of the sparganum is extremely vigorous and muscular, and closely resembles that of the adult. Ovoid calcareous corpuscles appear as transparent, ovoid, lamellated granules scattered randomly throughout the scolex, apart from the tip. No gland cells could be detected within the scolex.

In the presence of neutral red, the scolex granually became uniformly stained; again, no gland cells were delineated. No secretion of material from within the scolex to the exterior was detected.

(b) Histology of the sparganum scolex

See Figs. 7,8 and 9

The external surface of the sparganum scolex is covered by microtriches 2.3 μ long. These microtriches arise from the tegument which is about 10 μ thick. Under the light microscope the tegument appears fairly homogenous and clear (Fig. 6,D). Immediately below the tegument are the tegumental cells which appear as a single layer of closely packed cells with darkly staining nuclei. Their cytoplasmic connections with the distal cytoplasm are just faintly discernible under the light microscope (see Fig. 6,D).

Below the tegument and comprising most of the scolex is the parenchyma. The cells of the parenchyma are irregular in shape and size and cell membranes are not distinct; the parenchyma thus appears syncytial with nuclei randomly scattered within it. Calcareous corpuscles about 10μ-15μ in diameter, appear irregularly throughout the parenchyma (see Fig. 6,A,B and C).

A conspicuous ring of longitudinal muscles lies about within the parenchyma (see Fig. 6,A and B). Enclosed by these muscles and distributed randomly, are irregularly shaped masses of material, referred to here as amorphous bodies. These range in size from 10μ-20μ. The amorphous bodies appear to be extracellular deposits since nuclei could not be detected within them. Sometimes these bodies are not distinctly demarcated and they merge gradually with the parenchyma. They are composed of masses of granules which stain a reddish-orange with Heidenhain's Azan technique.

Two lateral excretory canals, about 10μ in diameter each, may be observed in cross-sections of the middle and posterior parts of the scolex. In the more anterior regions the excretory canals anastomose. Conspicuous nerve cords are present lateral to each of the excretory canals.

FIGURE 10

Table illustrating results of histochemical tests on the sparganum scolex.

+++	heavy positive reaction
++	medium positive reaction
+	light positive reaction
0	negative reaction
?	generalised diffuse reaction

(for abbreviations used for Methods see p.32 & 33)

Method	Sites in the scolex							
	D	T	P	M	E	N	C	A
PAS	+++	+	+	++	0	0	++	+++
Glycogen	0	0	++	++	0	0	0	0
AB	0	0	0	0	0	0	0	0
Toluidine Blue*	0	0	0	0	0	0	0	0
Sudan Black B	+++	0	0	+	+	0	++	+
HgBPB	++	+	0	+++	++	+	++	+++
MGP	0	+++	+	0	0	0	0	0
Acridine Orange	0	+++	+	0	0	0	0	0
Esterase	?	?	?	?	?	?	?	?
LAP	0	0	0	0	0	0	0	0
Acid Phosphatase	0	0	0	0	0	0	0	0
Alk. Phosphatase	+++	++	0	0	0	0	0	0

*Metachromasia

D=distal tegument
T=tegumental cells
P=paranchyma
M=muscles
E=excretory canals
N=nerves
C=calcareous corpuscles
A=amorphous bodies

No gland cells, which might be involved in secreting material to the exterior, could be detected in the scolex (see Fig. 3,C and D).

(c) Histochemical study of the sparganum scolex

Fig. 10 presents the results of the histochemical tests on the sparganum scolex of S. erinacei. No differences could be detected between scoleces of spargana taken from infected cane toads and those which had recently penetrated the gut of mice, and the following remarks apply equally to spargana recovered from both sources.

(i) Distribution of non-enzymatic substances.

The Sudan black B method for lipids gave a strong reaction in the tegument. Small amounts of lipid were present in the muscles, excretory canals, and the amorphous bodies mentioned earlier. Slightly larger amounts were associated with the calcareous corpuscles (see Fig. 12,A and B).

For carbohydrates, the PAS reaction was strongly positive in the tegument (Fig. 11,A) and smaller amounts of carbohydrates were evident in the muscles, amorphous bodies and calcareous corpuscles. A very light and diffuse reaction was also observed in the parenchyma and subtegument. The PAS reaction in the parenchyma and muscles

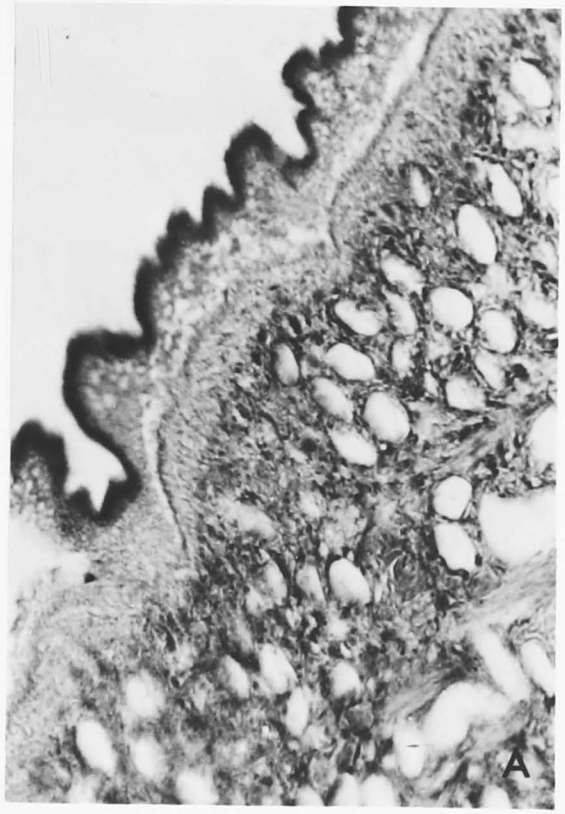
FIGURE 11

A - Test for carbohydrates (PAS). Note strong reaction in distal tegument. x500

B - Test for proteins (HgBPB). Strongest reaction in amorphous bodies and tegument. x50

C - Test for nucleic acids (MGP). Strong reaction for RNA in perinuclear cytoplasm of tegumental cells. x50

D - Control section; after action by RNAase (MGP). x50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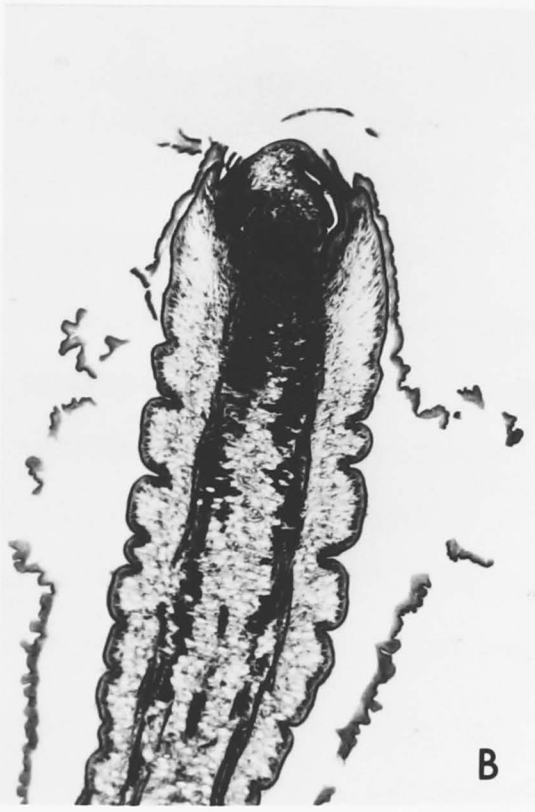

B


C


D

was clearly due to the presence of glycogen since it was abolished following diastase treatment. PAS reactions in other locations however persisted after diastase and thus showed that other types of polysaccharide were present in the sparganum scolex. (see Fig. 12,C and D). The AB test was negative and there was no evidence of metachromasia with the toluidine blue test, thus showing that acid mucopolysaccharides were absent. Thus, the mucopolysaccharides were neutral mucopolysaccharides.

The HgBPB test stained almost the whole section of the scolex except for negligible amounts in the parenchyma. The muscles and amorphous bodies gave especially strong reactions indicating higher concentrations of protein in these regions (Fig. 6,A and B; 11,B).

Tests for DNA and RNA were carried out using the MGP method and the acridine orange techniques. DNA was demonstrated in the nuclei in the scolex. RNA was seen to be almost totally concentrated in the cytoplasm of the tegumental cells with only small scattered amounts in the parenchymal cells. Ribonuclease destroyed RNA activity (Fig. 11,C and D). It was noted, with the acridine orange technique, that the normal green fluorescence seen in nuclei due to DNA was masked by intense red fluorescence

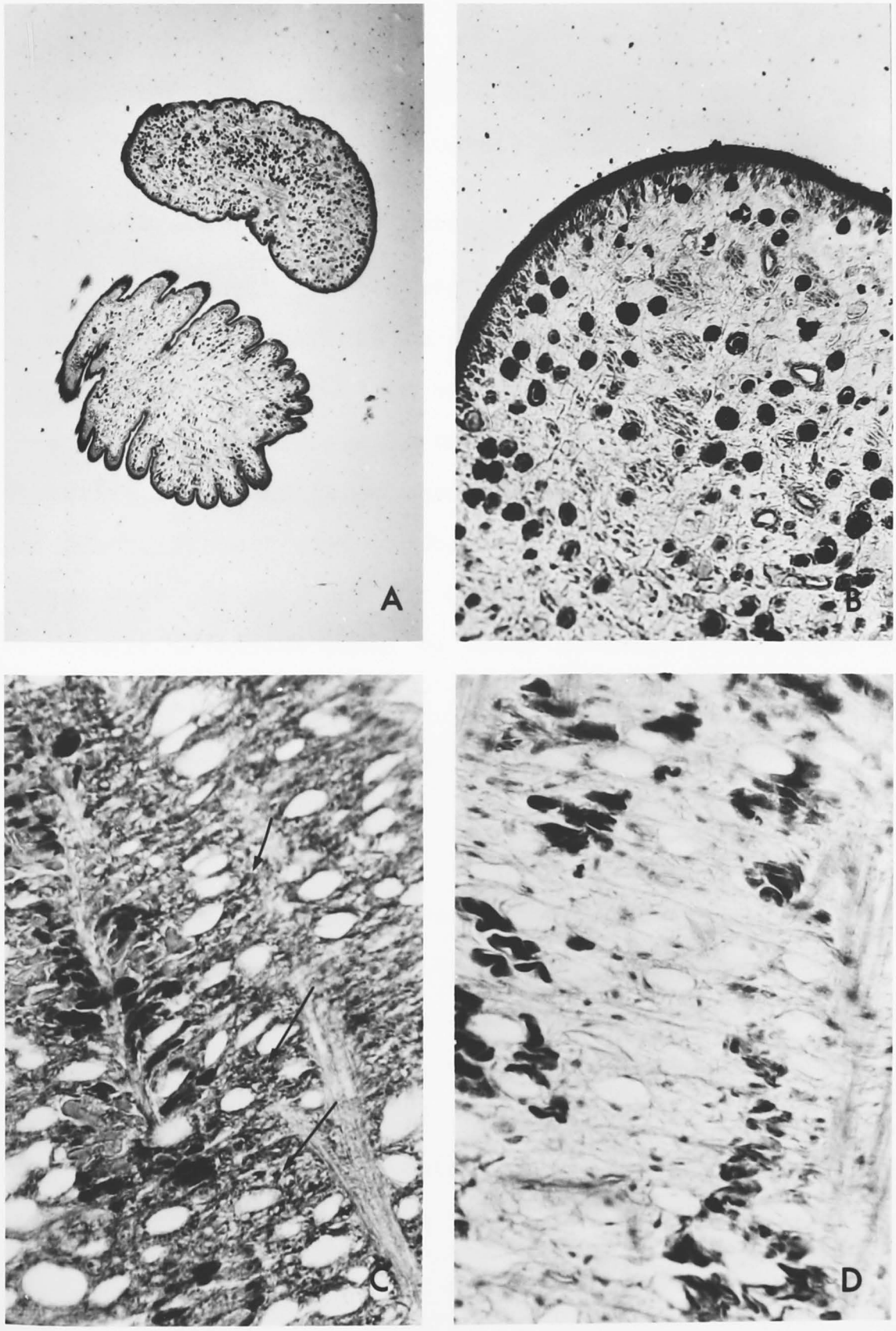
FIGURE 12

A - Test for lipids. (Sudan Black B) x100

B - As above. Note strong reaction in tegument and calcareous corpuscles. x200

C - Test for glycogen (PAS). Arrows show glycogen granules in parenchyma. Dark areas are the amorphous bodies. x500

D - Control after diastase action (PAS). Note absence of glycogen granules in parenchyma. Persistent reaction in amorphous bodies indicate presence of other polysaccharides. x500


A
B
C
D

of RNA. The green fluorescence became visible, however, following ribonuclease treatment.

(ii) Distribution of enzymatic substances.

The tegument and the tegumental cells of the sparganum scolex showed a very distinct reaction for alkaline phosphatase (see Fig. 13,A and B). There was no apparent reaction for the enzyme in any other regions. False positive reactions (i.e. persistence of reaction product in controls) were seen in the calcareous corpuscles and these were probably due to the presence of insoluble inorganic phosphate known to occur in these structures (von Brand, 1966).

Acid phosphatase was absent in the scolex of the sparganum (Fig. 13,C and D). Again, a false positive reaction was given by the calcareous corposcles. The test for leucine amino-peptidase was negative in the sparganum scolex. The limited number of tests for esterase which were carried out gave inconclusive results. A weak and diffuse reaction was given throughout entire sections of the scolex, and no localised concentrations of reaction product were detected.

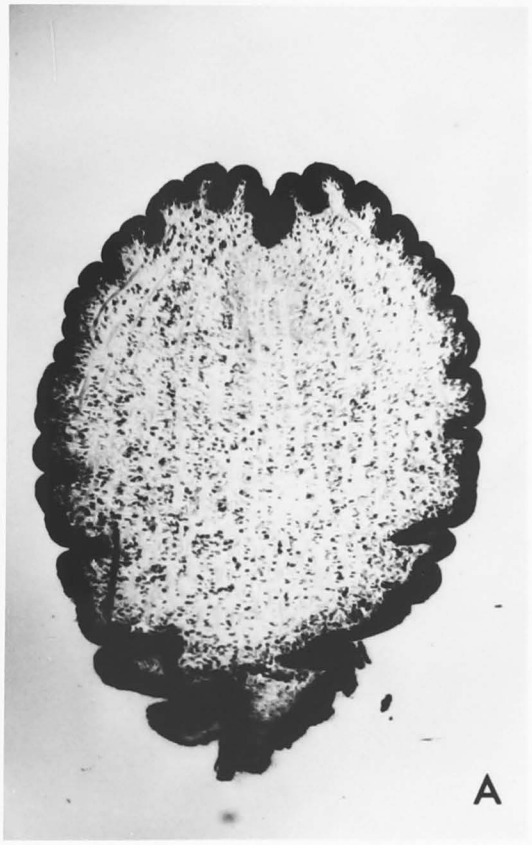
FIGURE 13

A - Test for alkaline phosphatase (Gomori). Strong reaction in tegument; reaction in calcareous corpuscles is a false positive probably due to reaction with inorganic phosphate deposits. x100

B - Control for above; note persistence of reaction in calcareous corpuscles. x200

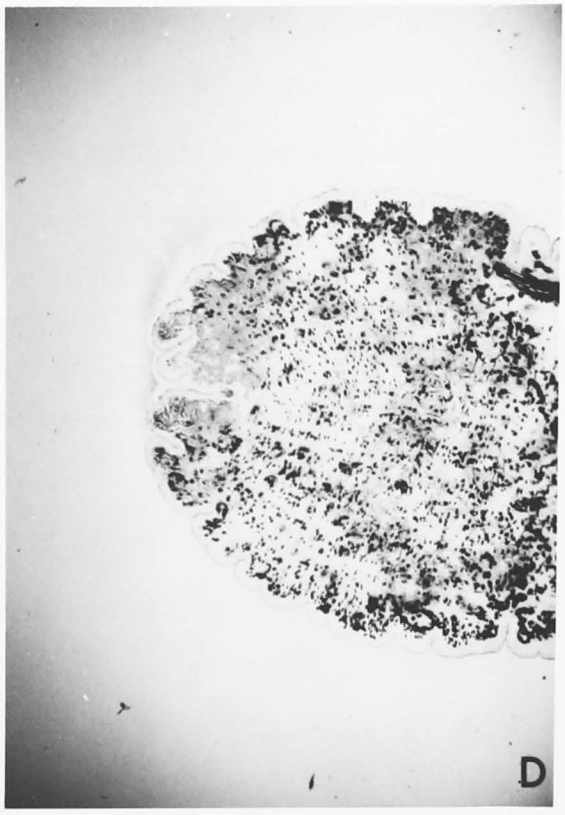
C - Acid phosphatase test (Gomori). Negative except again for false positive in calcareous corpuscles. x100

D - Control for C; false positive again persistent. x100


A


B


C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DISCUSSION

From the results in this chapter, the structure of the sparganum scolex appears to be relatively simple. Apart from the muscles, nerves and excretory canals, the scolex is filled with parenchymatous tissue. Calcareous corpuscles and other deposits in the form of amorphous bodies are also present in the parenchyma but no specialised gland cells were detected. No secretion from the scolex was detected in living spargana.

The rationale for this examination of the scolex of the sparganum of *S. erinacei* for glands, as mentioned in the introduction, was to find if glands and glandular secretions were present which might facilitate the process of penetration by the sparganum through the gut wall of the intermediate host. That glands might be involved was suggested by Smyth and Heath (1970) who stated that "Presumably, the cephalic glands present in the plerocercoid are histolytic in nature, although their nature does not appear to have been investigated." However, as far as can be determined, glands have not been previously described in the plerocercoid of *S. erinacei* or any other species of *Spirometra*. The "cephalic glands" mentioned by Smyth and Heath (1970) were probably the frontal

glands of the plerocercoids of Diphyllobothrium osmeri, D. latum, D. vogeli and D. dendriticum described by Kuhlow (1953).

These glands predominate in the medullary layer and vary in distribution and number in the different species he described. Furthermore, Kuhlow (1953) noted that the glands had a crystalline content and discharge to the exterior by means of tortuous canals. The function of these glands, Kuhlow suggested, was to enable the plerocercoid to digest host intestinal tissue during penetration of the fish host. He cited as evidence that they were involved in this process the fact that adult individuals of D. osmeri, D. vogeli, D. dendriticum and D. latum do not possess the glands; if they were involved only in penetration they would presumably be unnecessary in the adult.

From this study of the anatomy of the scolex of the S. erinacei sparganum, the only structures which resemble the glands described by Kuhlow (1953) are the amorphous bodies described earlier. These bodies appear in cross-sections to be roughly in the same position in the plerocercoid scolex and to also have the same irregular pattern and size as indicated in Kuhlow's figures. This

is especially true in the case of D. latum in Fig. 9, p. 201. It was at first thought that these bodies in the sparganum scolex of S. erinacei were in fact glands analogous to those described by Kuhlow (1953). Results of studies described in this chapter, however, suggest strongly that they are not glands but rather extracellular deposits of organic material.

In the first place, the bodies do not have a discrete cellular structure and no nuclei have been seen within them. The bodies appear amorphous and irregular and frequently blend into the surrounding parenchyma. On staining with the Heidenhain Azan technique, these bodies appear reddish-orange in colour. The glands described by Kuhlow appeared bright blue in colour when stained by the same technique. Secondly, no canals, or connections of any kind, could be detected which connected these bodies to the exterior. Thirdly, these bodies also appear further down the length of the sparganum and are not limited to the scolex. Fourthly, no reduction in size, or change in their histochemical properties, could be detected in these bodies after the sparganum had penetrated the mouse host. Fifthly, no RNA was detected in these bodies which indicated that they are unlikely to be

involved in protein synthesis. Sixthly, from the results of Chapter 5, it is clear that although proteolytic enzyme(s) could be demonstrated around the tegument of the scolex, no proteolytic activity was found within the medullary region of the scolex where the amorphous bodies occur.

The function of these amorphous bodies is not known. They contain substantial amounts of neutral mucopolysaccharide and protein but relatively little lipid. One possible explanation is that they are deposits of excretory products possibly inactivated by association with protein. The sparganum lives within the tissue of the host and is therefore in direct and intimate contact with the host. The parasite might be expected, therefore, to minimise the amount of antigenic material being released into the host tissue so as not to provoke a strong host reaction against it. From the results of the penetration experiments in Chapter 2, it was evident that the sparganum can remain in the mouse host up to two months without causing any significant inflammatory reaction. For an internal parasite the size of the sparganum, this would be unexpected if its metabolic products were released into host tissues. It is worth noting in support of this view that when the adults

are established in an "external" environment such as the intestine, the amorphous bodies are no longer present in the scolex.

Thus it seems certain that glands similar to those described by Kuhlow (1953) are not present in the sparganum of S. erinacei. Takahashi (1959b) in a fairly detailed study of the histochemistry of the plerocercoid of S. mansoni also failed to mention glands in the scolex or deposits comparable to those described here. Li (1929), in a study of the life history of S. erinacei, described "histolytic glands" in the procercoid stage but did not describe any glands or amorphous bodies in the plerocercoid stage.

The absence of glands visible with the light microscope could mean that there are no histolytic glands of any kind present in the sparganum, thus implying that the mechanism of penetration is by some means other than with glandular enzymes. It could also mean that enzyme-producing cells may be structurally organised in such a way that they are not visibly apparent as glands under the light microscope. The results in the following chapters indicate that the latter case is more likely, since proteolytic enzyme(s) have been found in the scolex of the sparganum.

At this stage it is uncertain why distinct glands are present in the procercoid but not in the plerocercoid of S. erinacei. It is certain, however, that there is great variability among species of Pseudophyllidea since in D. osmeri, D. vogeli, D. dendriticum and D. latum, glands are present in the plerocercoid but not in the adults (Kuhlow, 1953), whereas glands are present in the adults of Dibothriocephalus wilsoni, Adenocephalus pacificus, Glandicephalus antarticus (Wardle and McLeod, 1952) and Abothrium gadi (Williams, 1960).

Takahashi (1959b) detected both acid and alkaline phosphatase in the "cuticle and subcuticle cells" of S. mansoni spargana. In this study of S. erinacei spargana, only alkaline phosphatase was detected. All the other histochemical results obtained for S. mansoni by Takahashi (1959b) agree substantially with those for S. erinacei, however.

Findings described by Saraki (1961) for the distribution of DNA and RNA in the plerocercoids of S. mansoni also agree with those for S. erinacei spargana as found in this study.

The presence of large amounts of RNA in the tegument,

and the PAS positive material in the distal tegument, may be related to the proteolytic enzyme(s) found in the same region (see Chapter 5). Further discussion on the importance of this will be described later in Chapter 6.

CHAPTER 4

THE FINE STRUCTURE OF THE TEGUMENT OF THE SPARGANUM SCOLEX OF SPIROMETRA ERINACEI

INTRODUCTION

Studies on the ultrastructure of the tegument of various cestodes have shown that the tegument is a complex living structure which is probably of great importance in the physiology of the worm. A number of different structures and organelles have been described (see Lee, 1966 for a review) but their exact functions are not known. In almost all previous studies the main interest has been in the nutritional and metabolic aspects of the tegument, and functional considerations of the structures and organelles present have been viewed in terms of absorption and transport of material into the worm.

There is now increasing evidence that the cestode tegument may also be involved in the synthesis and secretion of proteinaceous material to the exterior of the worm. Lumsden (1966a,b) suggested that structural and enzymic proteins which occur in the tegument of various

cestodes are synthesised in the "sub-cuticular cytoplasm" and then transported in quanta into the matrix of the external tegument. Some of this material may then be released from the tegument as secretory material.

Despite the apparent absence of secretory glands in S. erinacei (see Chapter 3), homogenates of the sparganum have been shown to possess proteolytic activity (see Chapter 5). Furthermore, the site of this activity is located in the tegument of the scolex (see Chapter 5). The tegument in this region is rich in protein and strongly PAS positive, and protein synthesis takes place in the tegumental cells as evidenced by the presence of substantial amounts of RNA in their cytoplasm (see Chapter 3). These results suggest strongly that the tegument could be involved in the synthesis of the proteolytic enzyme(s) noted above.

The plerocercoid or sparganum of S. erinacei is well known for its migration through host tissue. It is not inconceivable that the proteolytic enzyme(s) synthesised in the tegument are also released to the exterior, to facilitate this process. Thus, Lumsden's (1966a,b) view of protein transport to the exterior of the tegument in a number of cestodes could well apply in the present case.

A study of the fine structure of the tegument of S. erinacei spargana was therefore undertaken in an attempt to obtain ultrastructural evidence in support of this hypothesis. The results reported in this chapter show that small organelles are present in the tegument which may be associated with the production and release of proteolytic enzyme(s). Although the fine structure of the tegument of both plerocercoid and adult stages of S. erinacei has been examined previously by Yamane (1968), some of the structures described here were not reported.

METHODS AND MATERIALS

Spargana of S. erinacei were removed from naturally infected toads (Bufo marinus) and fed to experimental mice (Quakenbush strain). The spargana were removed from the abdominal cavity of the mice 7 days later.

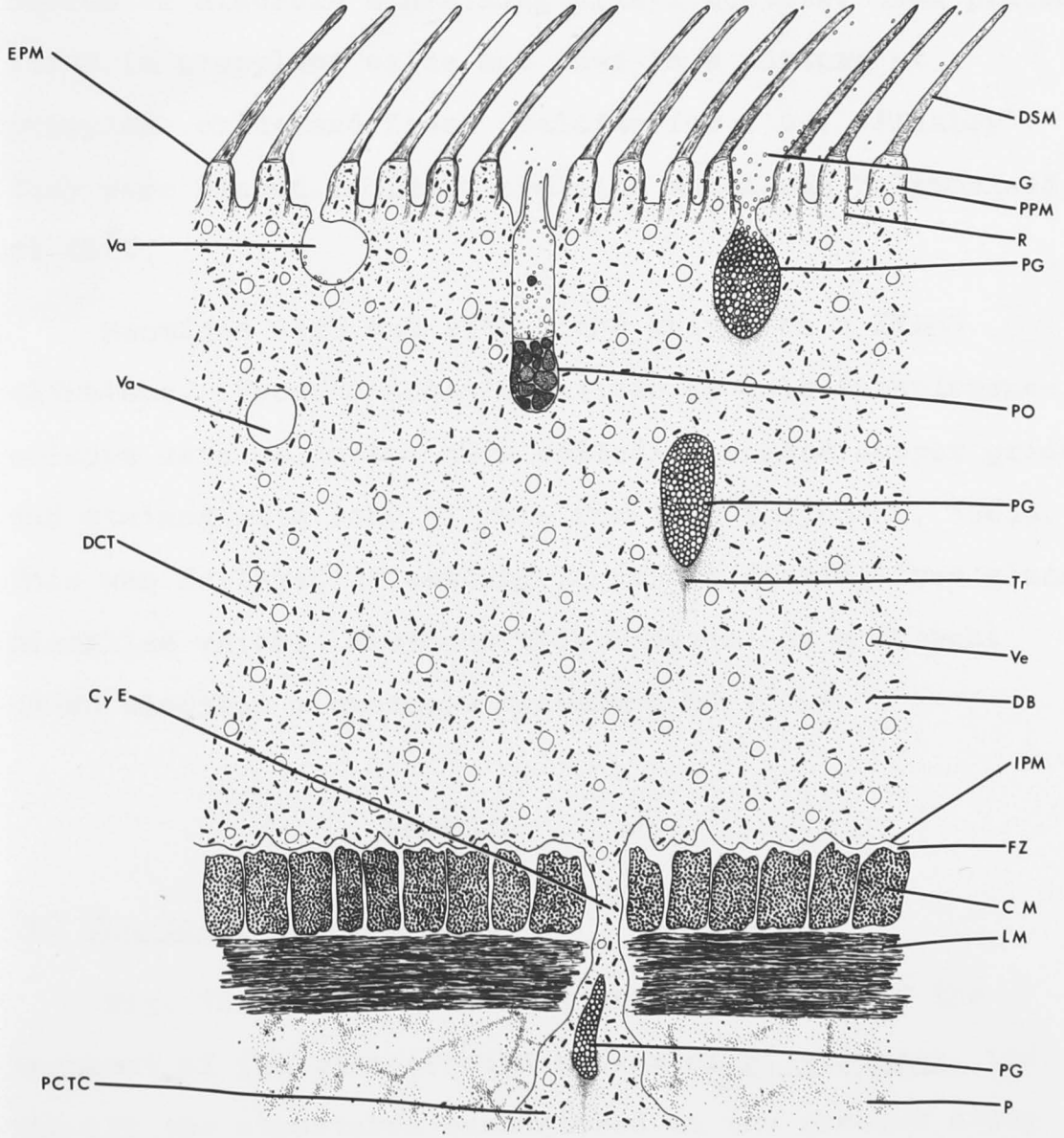
About 3-5 mm of the anterior region of each worm, i.e. the scolex, was cut off and the rest of the posterior portion discarded. Scoleces were immediately fixed in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.5) overnight at 4°C. They were then washed overnight in phosphate buffer to remove all traces of glutaraldehyde. The specimens were then placed in 2% OsO_4 in veronal acetate

FIGURE 14

Generalised diagram of the fine structure of the tegument. x10,000

List of abbreviations:

EPM-external plasma membrane; Va-vacuole; DCT-distal cytoplasm of the tegument; CyE-cytoplasmic extension; PCTC-perinuclear cytoplasm of the tegumental cell; DSM-distal shaft of the microthrix; G-granules (electron-transparent); PPM-proximal portion of the microthrix; R-"rootlet" of electron-dense material; PG-packet of granules; PO-pit organelle; Tr-"trail" of amorphous granular material; Ve-vesicle; DB-disc-like bodies; IPM-internal plasma membrane; FZ-fibrous zone; CM-circular muscles; LM-longitudinal muscles; P-parenchyma.


EPM
DSM
G
PPM
R
Va
PG
Va
PO
PG
DCT
Tr
Ve
Cy E
DB
IPM
FZ
C M
LM
PG
PCTC
P

buffer (pH 7.3) for 4 hr at 4°C and then rinsed several times in tap water. They were dehydrated in a graded series of alcohols containing uranyl acetate, then placed first in propylene oxide and then in a mixture of propylene oxide and fresh araldite for 1 hr. Finally they were placed in fresh araldite which was polymerised at 60°C.

Sections were cut with glass knives on a LKB-3 ultratome. Those displaying silver or gold interference colours were collected onto collodion coated copper grids and stained with lead citrate for 1 hr (Reynolds, 1963). This was followed by washing in carbon dioxide-free glass distilled water. Sections were examined in a Hitachi HS-HC electron microscope operating at 75 kV.

RESULTS

(a) The tegument - General

Fig. 14 is a diagrammatic representation of the tegument of the plerocercoid scolex of S. erinacei, indicating all the structures encountered in the present study and the names used for them. Where possible, the terminology used by Smyth (1969) is followed.

FIGURE 15

Microtriches at surface of tegument. Dark horizontal bar at bottom is a section fold. Note that the disc-like bodies are larger and more irregular in size here. Longitudinal strands in the distal shafts of microtriches discernible (micro-tubules ?). Arrows show an electron-dense coating over the external plasma membrane. x90,000

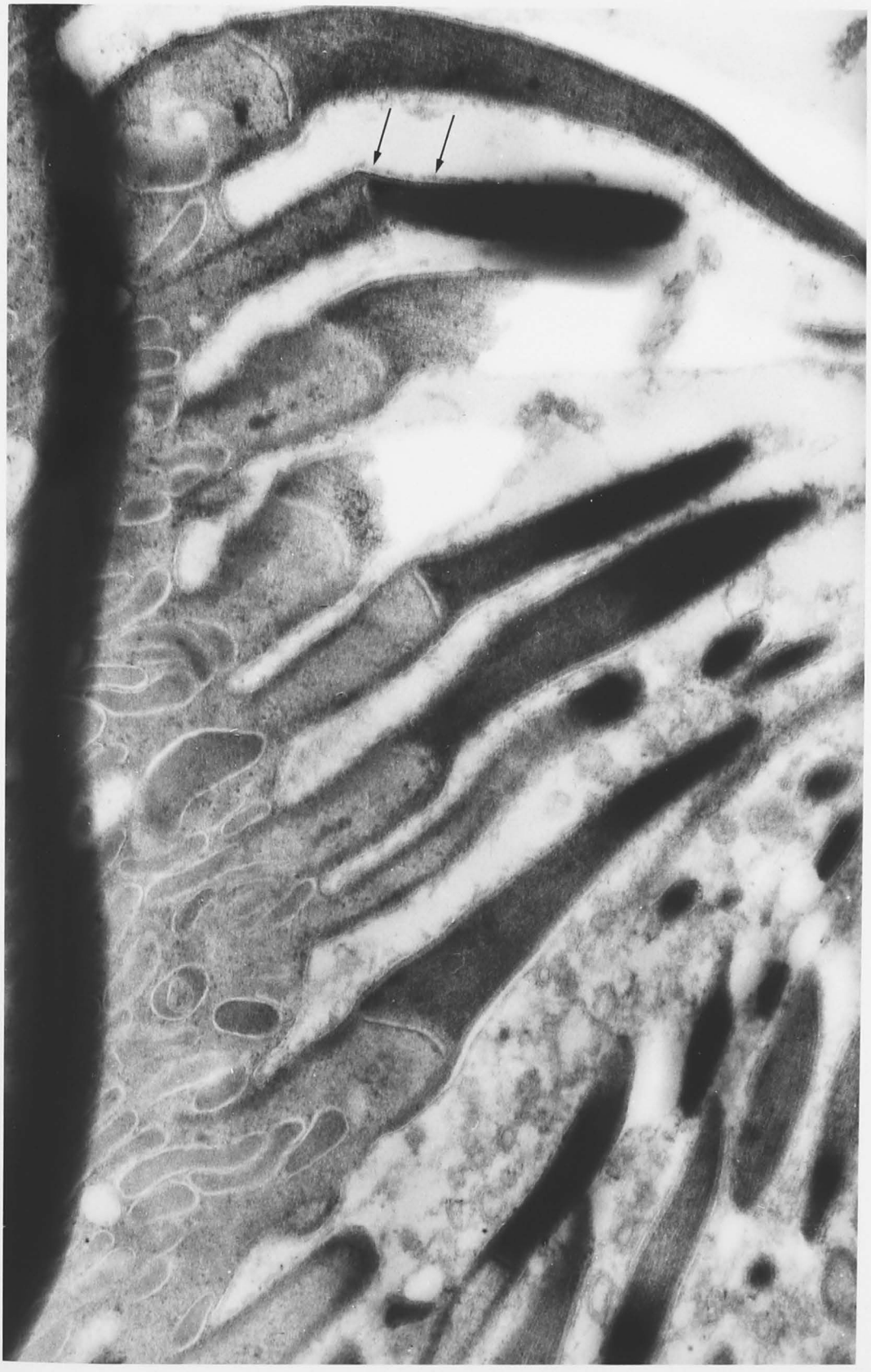
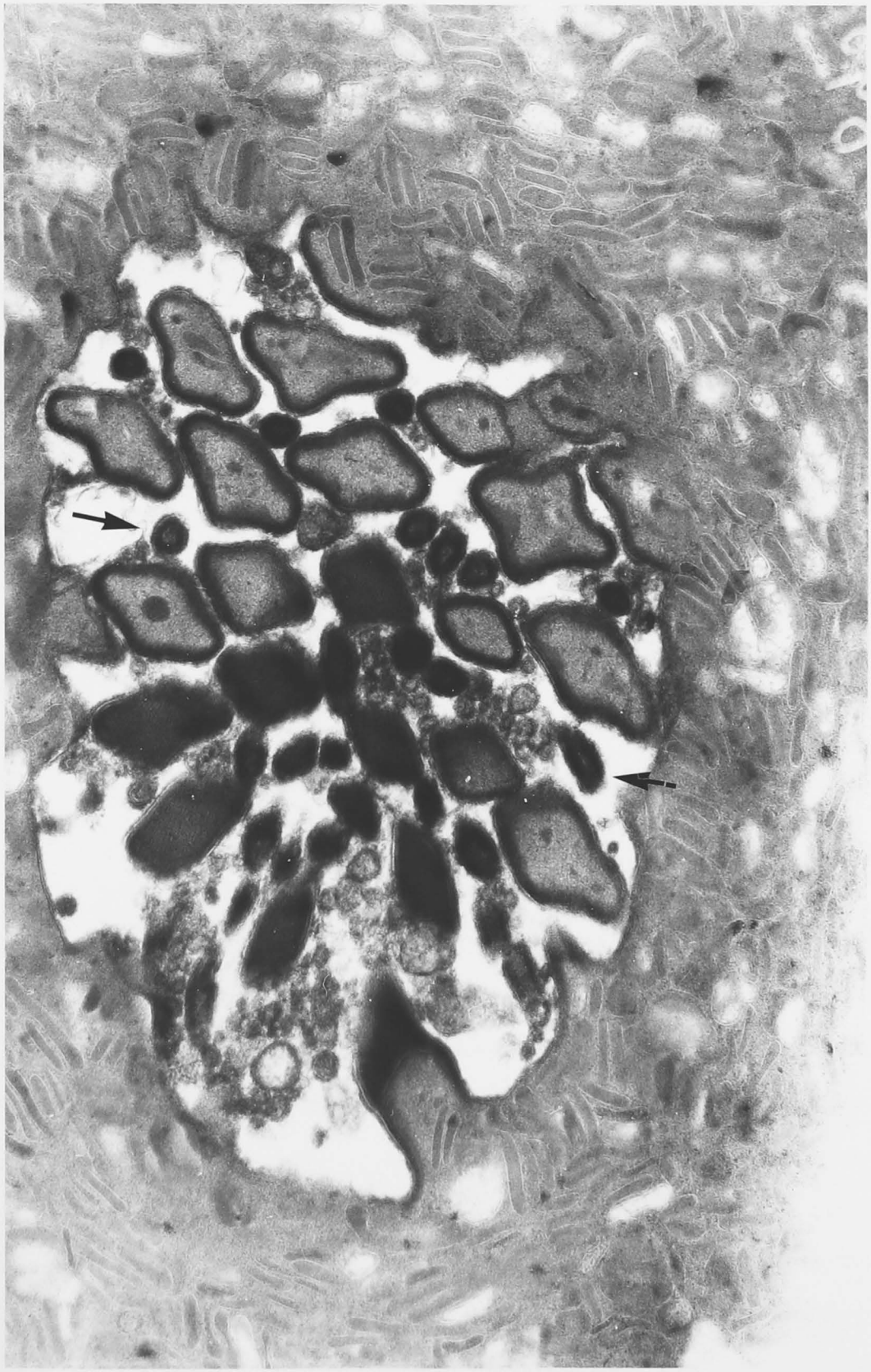


FIGURE 16

T.S. of microtriches. The rhomboid structure of the proximal portion of the microtriches is evident. Arrows show the cylindrical structure of the distal shaft. x70,000



Externally, the tegument is covered with microtriches which are approximately 2.3μ long and 0.3μ in diameter at the base (see Fig. 15). Each microthrix is divided into an electron-dense distal shaft and a clear proximal portion. The distal shaft is about 3-4 times the length of the proximal portion and is bent at an angle to it; a substructure of fine strands (micro-tubules?) is seen to run longitudinally in the distal shaft (see Fig. 15). A clear lucid zone at the base of the shaft separates the two portions of the microthrix. A ring of electron-dense material appears peripherally inside the proximal portion of the microthrix and extends down into the distal cytoplasm as "rootlets". This material is a continuation of the electron-dense material in the distal shaft of the microthrix. In cross-section, the basal portion of the microthrix appears rhomboid in shape whereas the shaft is cylindrical (see Fig. 16). An external plasma membrane covers the microtriches and is continuous over the whole surface of the tegument. This membrane is approximately 105 Å thick and is composed of two dense layers with a light band in between. An electron-dense discontinuous coating is faintly discernible over the external plasma membrane (see Fig. 15). Microtriches were observed to cover the entire surface of the tegument of the scolex including that lining the bothria.

FIGURE 17

Distal cytoplasm of the tegument. Note the infoldings of the internal plasma membrane; fibrous zone and muscle layers below. Structure seen probably represents section through nerve. x16,000

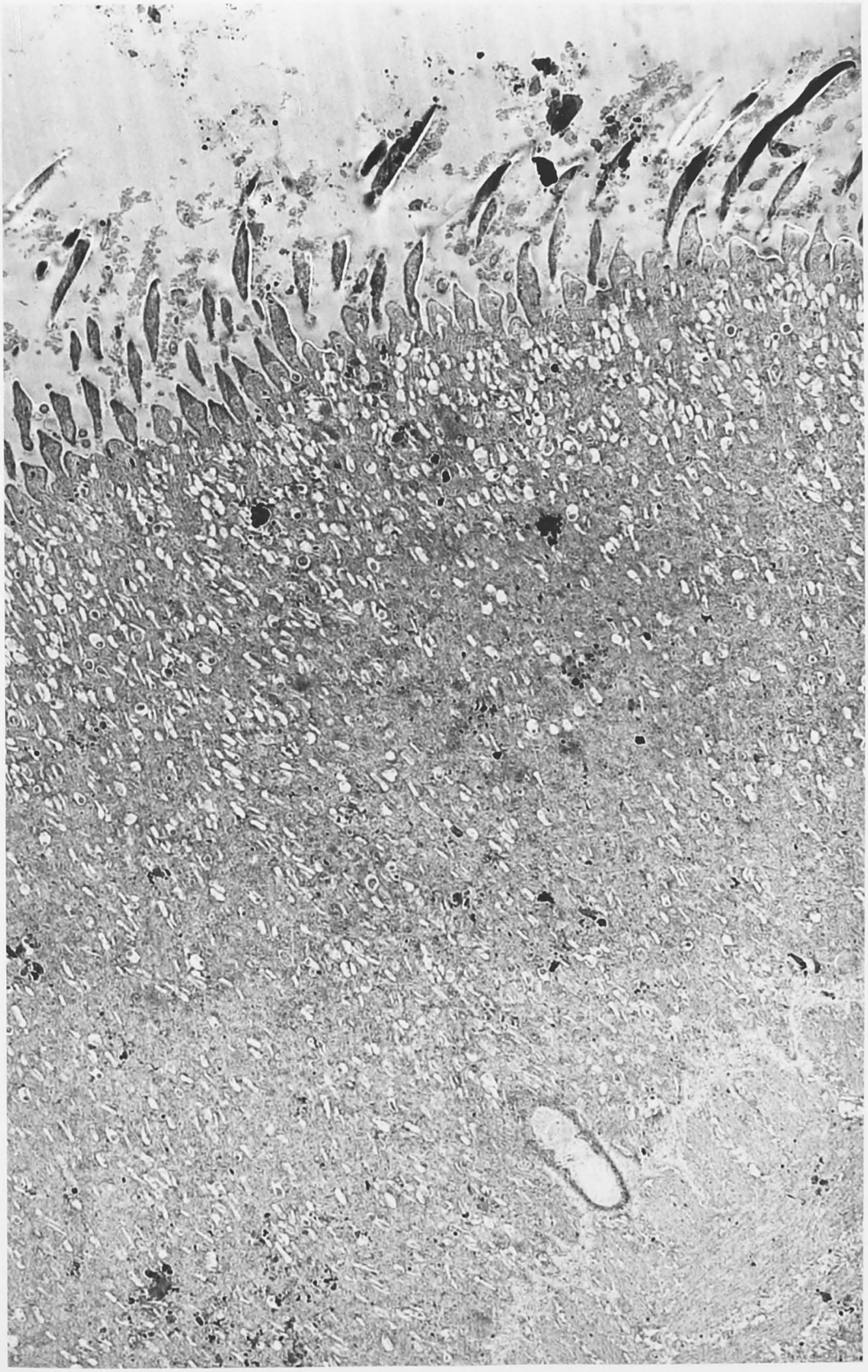
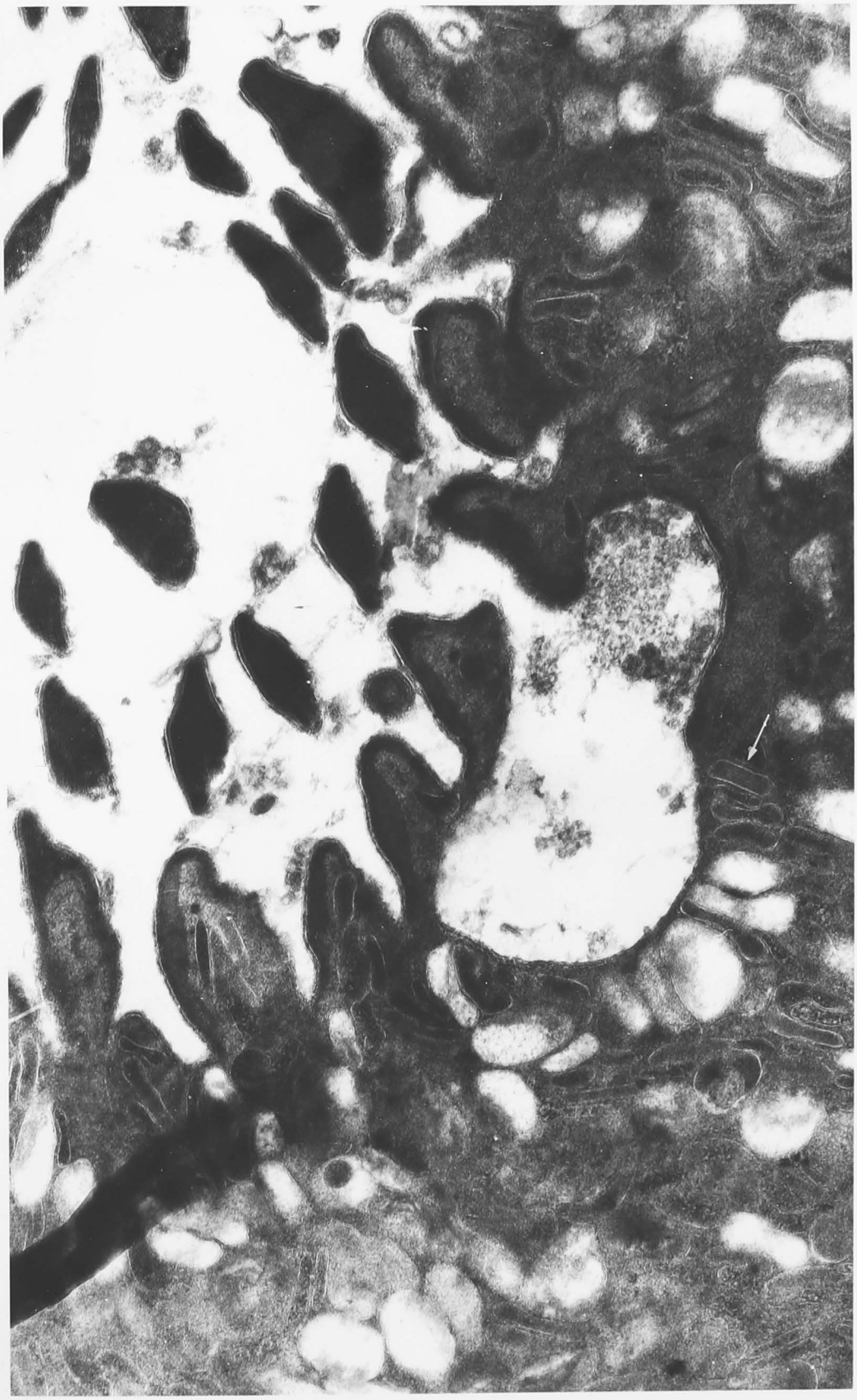


FIGURE 18

Vacuole with connection to exterior. Note presence of some granules within. Empty round structures, randomly distributed, are termed vesicles. Dark disc-like bodies are also scattered randomly in distal cytoplasm. Arrow shows striations in disc-like body. x100,000



Below the external plasma membrane is the distal cytoplasm which is composed of a granulated matrix with various cytoplasmic inclusions (see Fig. 17). The distal cytoplasm varies between 8μ and 12μ thick. Among the cytoplasmic inclusions are empty membrane-bound vesicles, usually about 0.2μ in diameter, which are randomly scattered in the matrix. These vesicles appear to be devoid of any contents (see Fig. 18). Among these vesicles, and appearing closely packed, are electron-dense disc-like bodies which are also membrane-bound. These are approximately 0.1μ in diameter as seen in Fig. 18. Sometimes however they appear much larger in size, up to 0.3μ in diameter, and more irregular in shape (see Fig. 15). Regular parallel light and dark bands often appear within cross-sections of the discs. Both the discs and the vesicles are evenly distributed throughout the distal cytoplasm.

Relatively large membrane-bound vacuoles, 0.6μ to 1.0μ in diameter, appear as sac-like involutions of the external plasma membrane. These are several times larger than the vesicles and small membrane-bound bodies can be seen within them (see Figs. 18, 19). Diffuse, electron-dense material is also present within the vacuoles. Some of the vacuoles lack connections with the exterior (Fig. 20).

FIGURE 19

Vacuole connected by narrow canal to the exterior between microtriches. Note the presence of membrane bound granules and diffuse electron-dense material within them. x120,000

FIGURE 20

Vacuole just below external plasma membrane, unconnected to exterior. x60,000

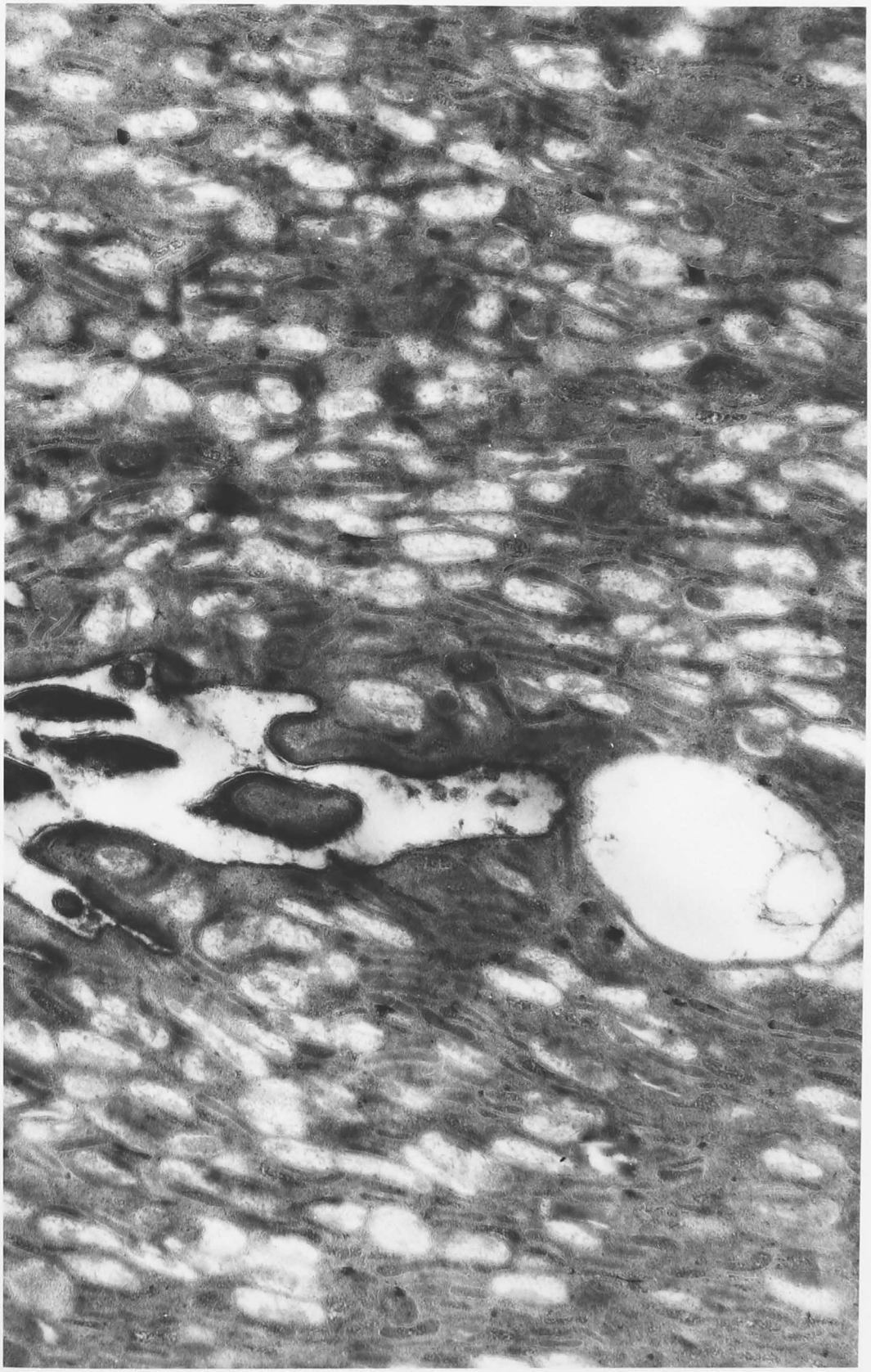


FIGURE 21

Another view of the distal cytoplasm of the tegument. Note the cytoplasmic extension passing through muscle layers to tegumental cell below (not shown). Note that internal plasma membrane is continuous with membrane of cytoplasmic extension. x16,000

It is possible that they may be formed as a result of pinocytosis.

Surprisingly, very few mitochondria were seen in the distal cytoplasm. A number of other structures which are present in the matrix of the distal cytoplasm will be described below.

The distal cytoplasm is limited basally by an internal plasma membrane which is partially infolded into the cytoplasmic matrix (see Fig. 17). This membrane is continuous with the cell membrane of the tegumental cell (see Fig. 21) so that the matrix of the distal cytoplasm is continuous, through cytoplasmic extensions, with the perinuclear cytoplasm of the tegumental cell.

Below the internal plasma membrane is a fibrous zone whose amorphous contents are continuous with the extracellular contents of the parenchyma. The muscle layers lie below this fibrous zone with the circular muscles external to the longitudinal muscles (see Fig. 17).

(b) The tegument - Organelles

Two basic types of organelles can be distinguished in the tegument of S. erinacei. These appear as if they may be involved in secreting material out of the tegument,

FIGURE 22

Diagram of pit organelle in the tegument. (Scale line = 1 μ)

List of abbreviations:
EPM-external plasma membrane; PPM-proximal portion of the microthrix; Ci-cilia-like structure; Ve-vesicle; DB-disc-like body; DG-electron-dense granule; G-granule (electron-transparent).

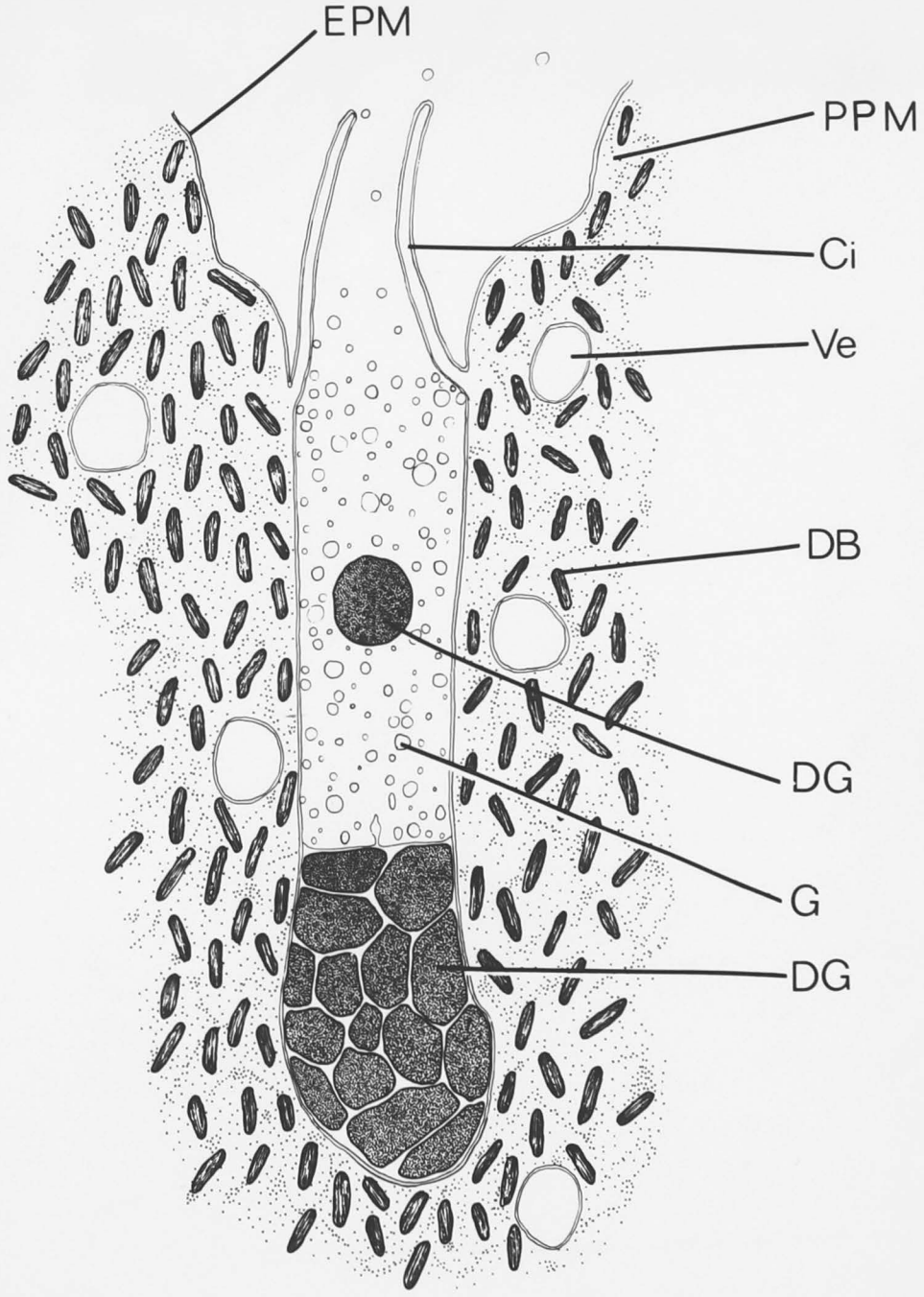
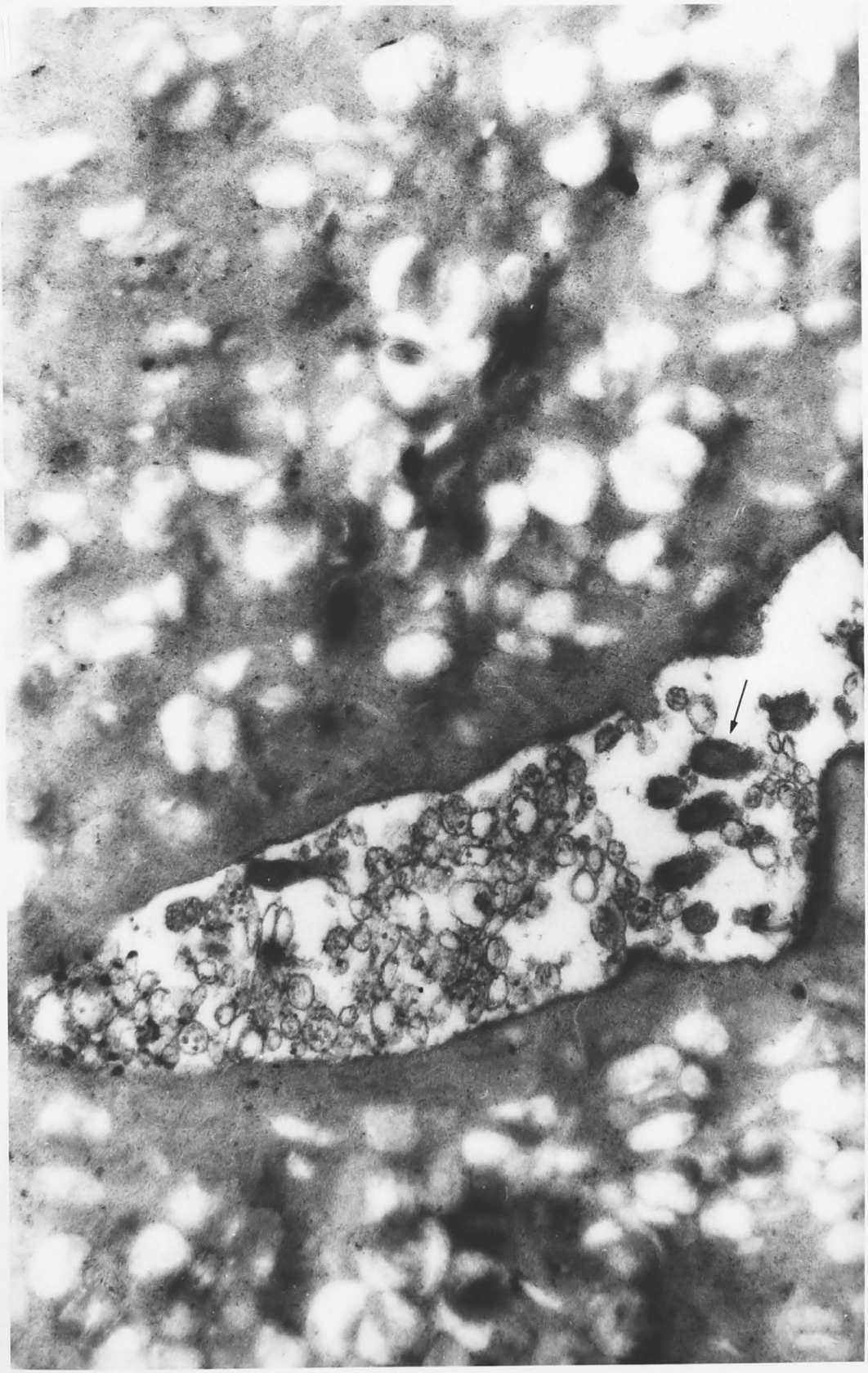

EPM
PPM
Ci
Ve
DB
DG
G
DG

FIGURE 23

Pit organelle. Dark spherical body in the centre is probably a loose electron-dense granule similar to the large electron-dense granules packed into the bottom of pit. Note mass of small transparent membrane bound granules in hollow of pit; white arrow shows one such granule probably being extruded from the electron-dense granules below. Black arrow shows cilia-like structures. x50,000

FIGURE 24

Pit cut diagonally, showing upper portion of organelle. Arrow indicates cilia-like structures. Note the presence of various sub-structures within the membrane bound granules. x70,000



and are therefore described here separately from the more common inclusions described in (a).

The first type of organelle occurs as a sunken pit at the surface of the tegument. The opening at the top of the pit is rather irregular and fans out to form a depression at the surface among the microtriches. Fig. 22 gives a diagrammatic representation of the structure of the pit. The main portion is composed of an essentially cylindrical lumen, 0.7μ in diameter; the entire pit is 3.4μ deep from its base to the level of the external plasma membrane, but about 2.3μ from its base to a ring of cilia-like structures which surround the cylindrical region of the pit distally (see Figs. 23, 24). The small cilia-like structures are about 0.07μ thick and circular in cross-section and fine fibrillar sub-structure is discernible in cross-section of them (Figs. 25 and 26 show transverse sections of the pit at various levels). The wall of the pit is continuous with the external plasma membrane, which envelops the cilia-like structures as well.

The basal third of the pit contains large, spherical to polygonal, tightly-packed, electron-dense granules, 0.2μ to 0.3μ in diameter, which appear to be membrane-bound. It is not certain if these electron-dense granules are

FIGURE 25

T.S. of pit in the middle. Round section shows the cylindrical structure of pit. Bodies within are membrane bound granules described earlier. x120,000

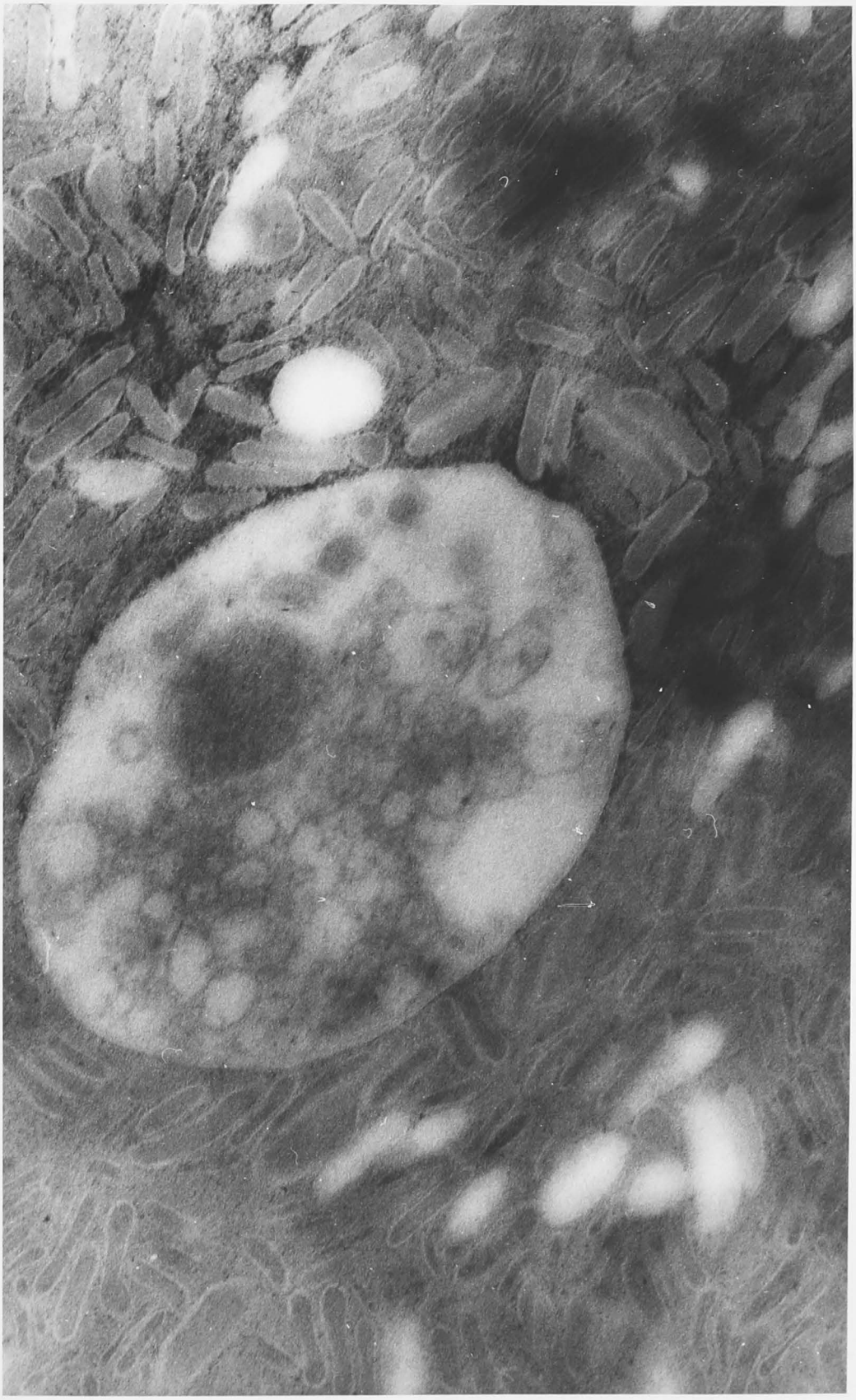
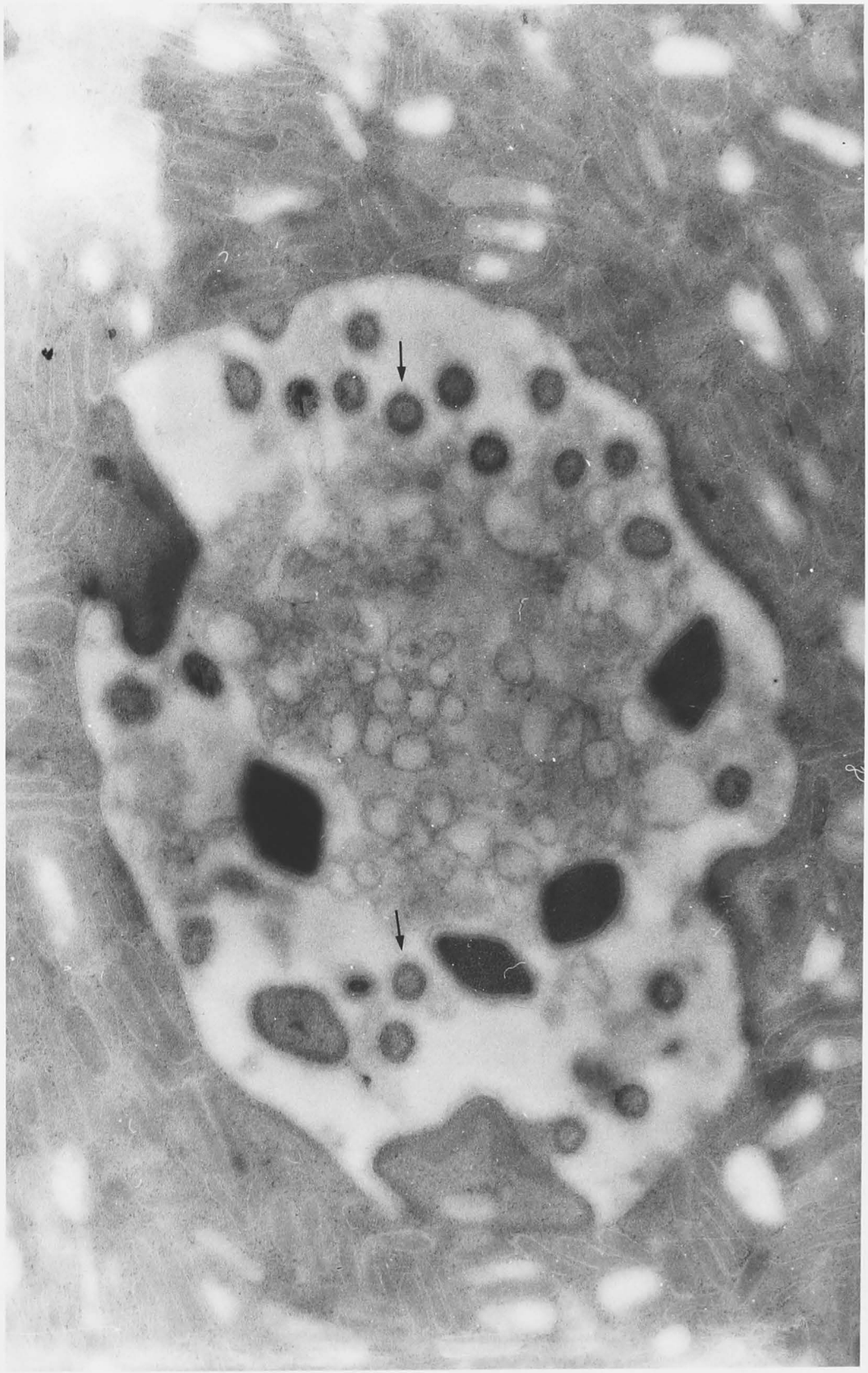


FIGURE 26

T.S. of pit at opening. Note transverse sections through microtriches. Arrows indicate sections of cilia-like structures near mouth of pit. x100,000



secreted to the exterior although Fig. 23 shows what appears to be one granule, released from the tightly-packed mass, in the lumen of the pit. Small membrane-bound bodies, approximately 0.06μ to 0.08μ in diameter, loosely fill the lumen of the organelle. Some of these bodies appear to have a membranous substructure. Many of them appear to be derived from the surface of the electron-dense mass of granules at the base of the pit (see Fig. 23). The ring of cilia-like structures, surrounding the lumen distally, appear to partly trap the small membrane-bound bodies; hence these granules appear as clusters below the ciliary ring. These pits in the scolex could only be located at or near the anterior tip of the sparganum.

The second type of organelle is more common than the pits described above (see Fig. 27). These organelles are packets of granules which are often found connected to the exterior by an opening between two microtriches (see Fig. 28). The granules enclosed in each packet are membrane-bound and electron-transparent, approximately 0.06μ to 0.08μ in diameter, and may possess membranous substructures. Similar membrane-bound granules are seen on the outside surface of the tegument (see Fig. 30) and this suggests these packets of granules are secreted to

FIGURE 27

Diagram of a packet of granules in the distal tegument with opening to the exterior. (Scale line = 1μ)

List of abbreviations:

EPM-external plasma membrane; DSM-distal shaft of the microthrix; EDC-electron-dense coating at the surface of the external plasma membrane; LZ-lucid zone of microthrix; PPM-proximal portion of the microthrix; R-"rootlet" of electron-dense material at the base of microthrix; G-granule (electron-transparent); PG-packet of granules; DB-disc-like body; Ve-vesicle.

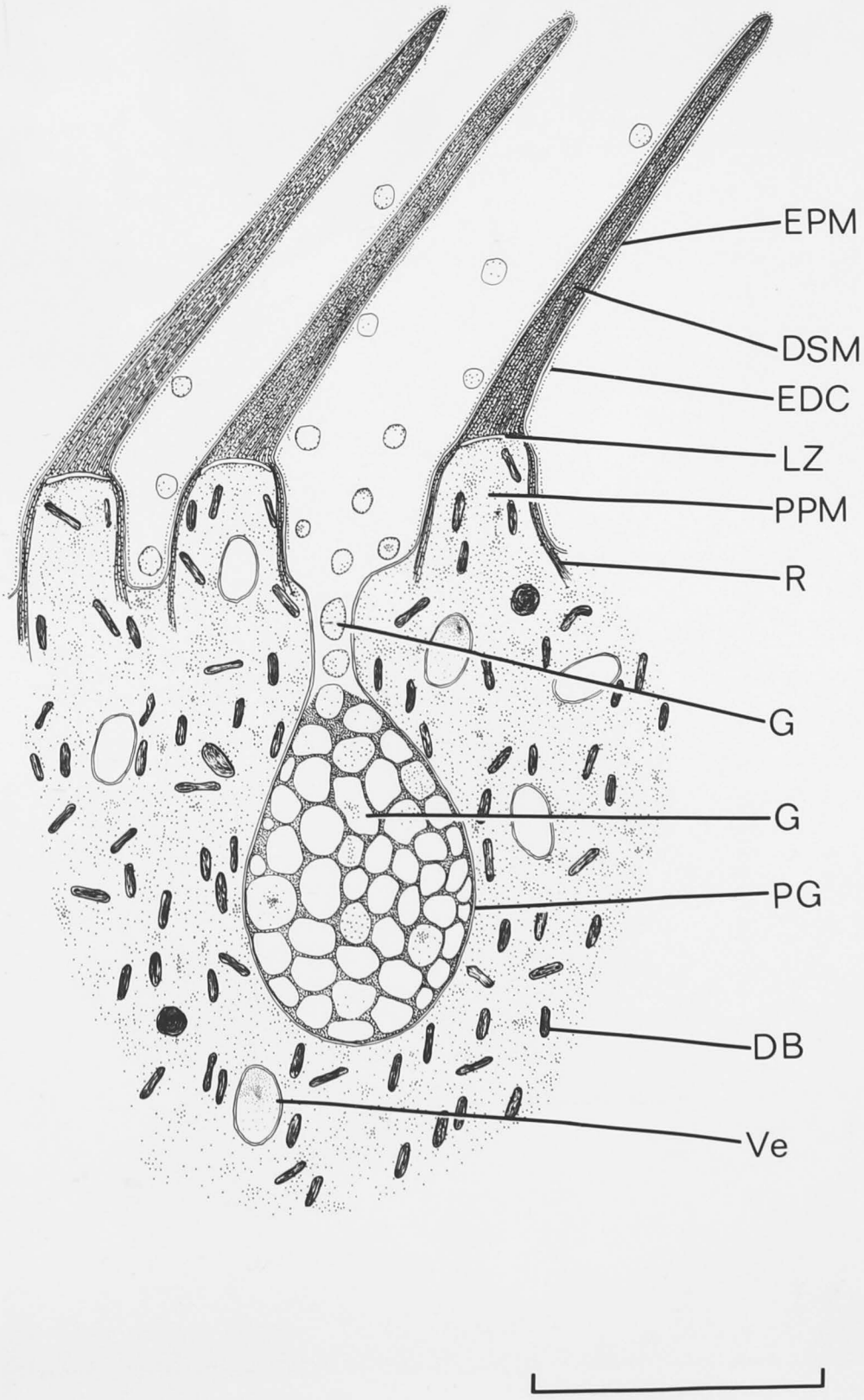
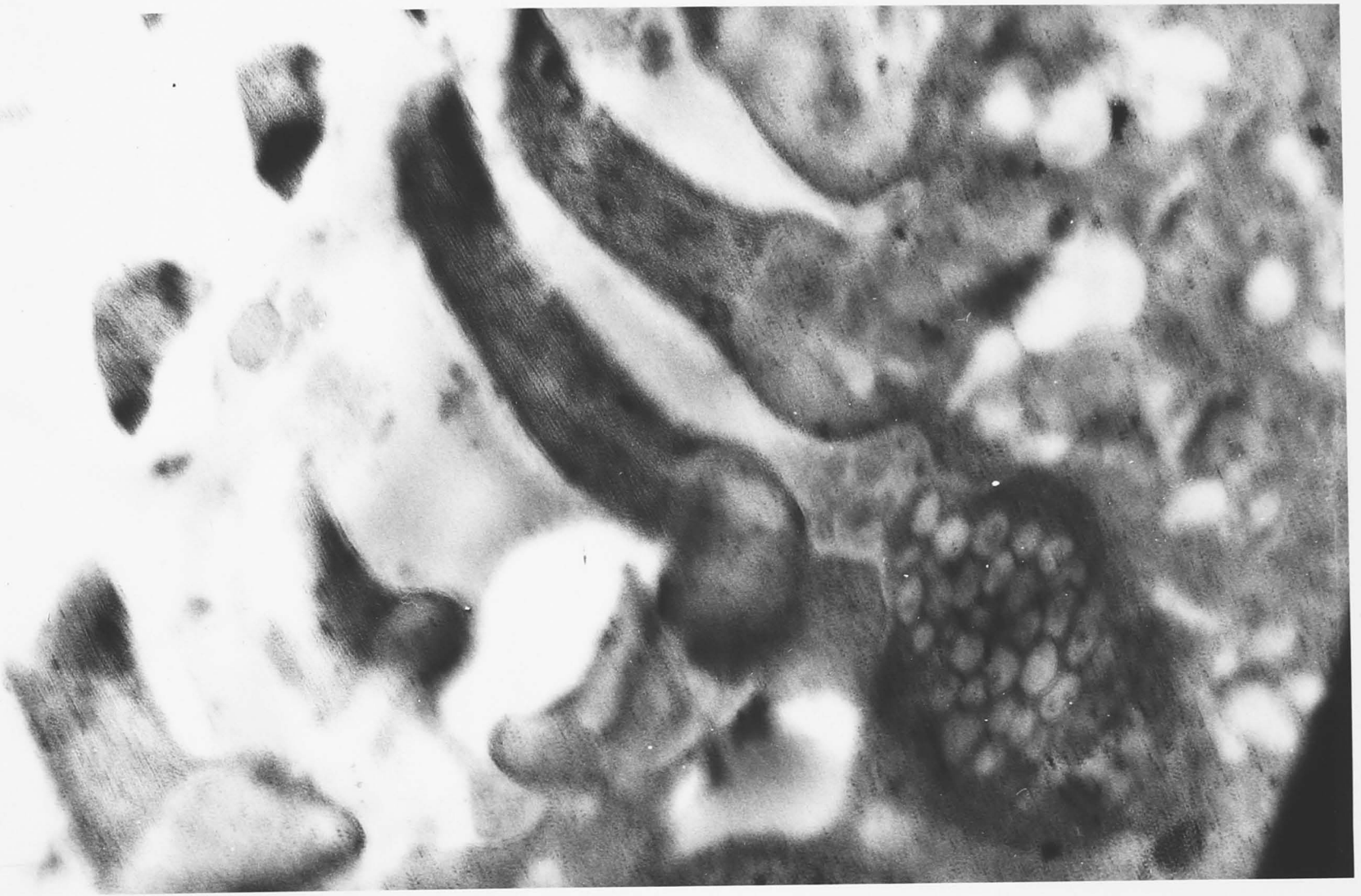

EPM
DSM
EDC
LZ
PPM
R
G
G
PG
DB
Ve

FIGURE 28

Two packets of granules opening to exterior. Note granules at openings and outside tegument; it is suggested that the granules are released. x100,000

FIGURE 29

Another section of packet of granules opening to the surface of tegument. x100,000



the exterior.

The packets of granules are generally oval or flask-shaped from 0.5μ to 1.2μ; the latter shape is marked in sections which pass through the openings to the exterior. In addition to the shape, the absence of a ciliary ring or electron-dense granules, make it possible to clearly distinguish these organelles from the first type. Sometimes, however, as in Figs. 30 and 31 there appears to be a distal protruberence present at the opening to the exterior. It is not known if this is a ciliary body associated with the organelle or a plug of material. As it is difficult to see a distinct connection between this structure and the organelle, the possibility of it being an artifact can not be discounted.

The packets of transparent membrane-bound granules in the distal cytoplasm which lack connection with the exterior, are sharply oval and tapered in shape with the sharper ends at right angles to the external plasma membrane (see Fig. 32). Fig. 33 shows that the packets themselves are made up of two unit-membranes completely enclosing the granules. Fig. 34 shows a number of these packets of granules within the matrix of the distal cytoplasm. They appear to vary in size and carry varying numbers of the membrane-bound granules. An amorphous,

FIGURE 30

Packet of granules opening to the exterior. Note large number of granules at surface. Arrow indicates distal protruberence sometimes present - their significance is uncertain. x55,000

FIGURE 31

Another packet of granules. Arrow indicates distal protruberence. x80,000

granular zone often surrounds the packets or is left as a proximal "trail" (see Fig. 35). This amorphous, granular zone merges with the cytoplasmic matrix and appears to be formed of the same ground substance but lacks the vesicles and disc-like bodies. The shape of the packets here, together with the presence of a proximal trail, suggest that the packets may be moving upwards in the distal cytoplasm and their contents are eventually discharged to the exterior.

Packets of membrane-bound granules also appear in the perinuclear cytoplasm of the tegumental cells below the fibrous zone (see Fig. 36). These packets are ovoidal to irregular in shape and appear to be associated with membranes in the perinuclear cytoplasm of the tegumental cell. The enclosed, granules are similar to those described above, but the relationship of these packets to those in the distal cytoplasm is not clear. However, Fig. 37 shows what appears to be a packet of granules in the region of the fibrous zone between the distal cytoplasm and the circular muscles, and its sharply tapering shape suggests that it is being squeezed through a cytoplasmic extension from the tegumental cell into the distal cytoplasm. It is thus possible that the more regular packets in the distal cytoplasm originate from the irregular packets

FIGURE 32

Packet of granules in distal cytoplasm of tegument, near surface. Note presence of membranous sub-structure within some granules. Packet tapers at right angles to the surface, giving impression of moving upwards. x100,000

FIGURE 33

Packet of granules in distal cytoplasm. Note that the enclosing packet is formed by two unit membranes. Note that many of the granules contain membranous substructures; some granules appear to possess an electron-dense "cortex". x100,000

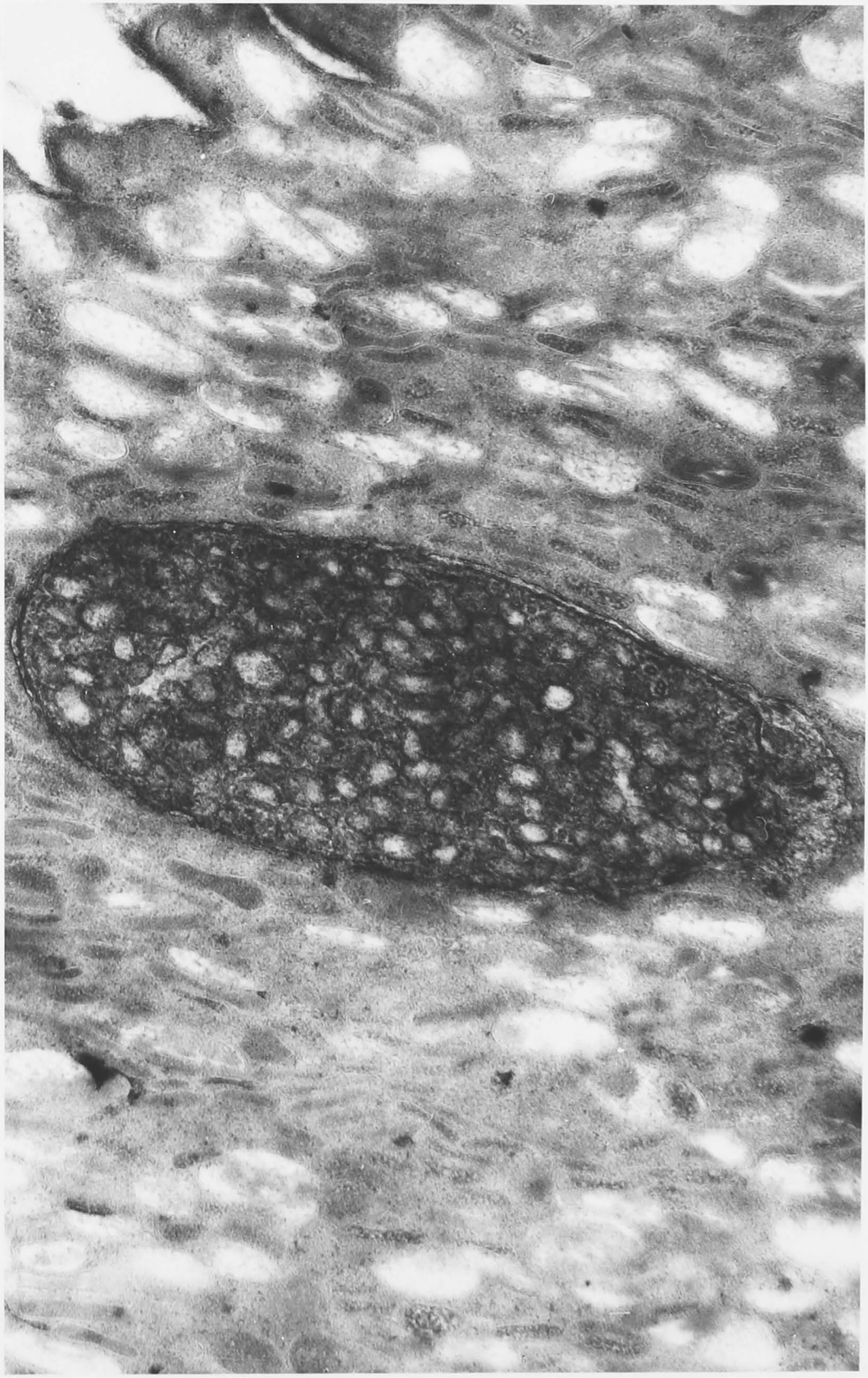
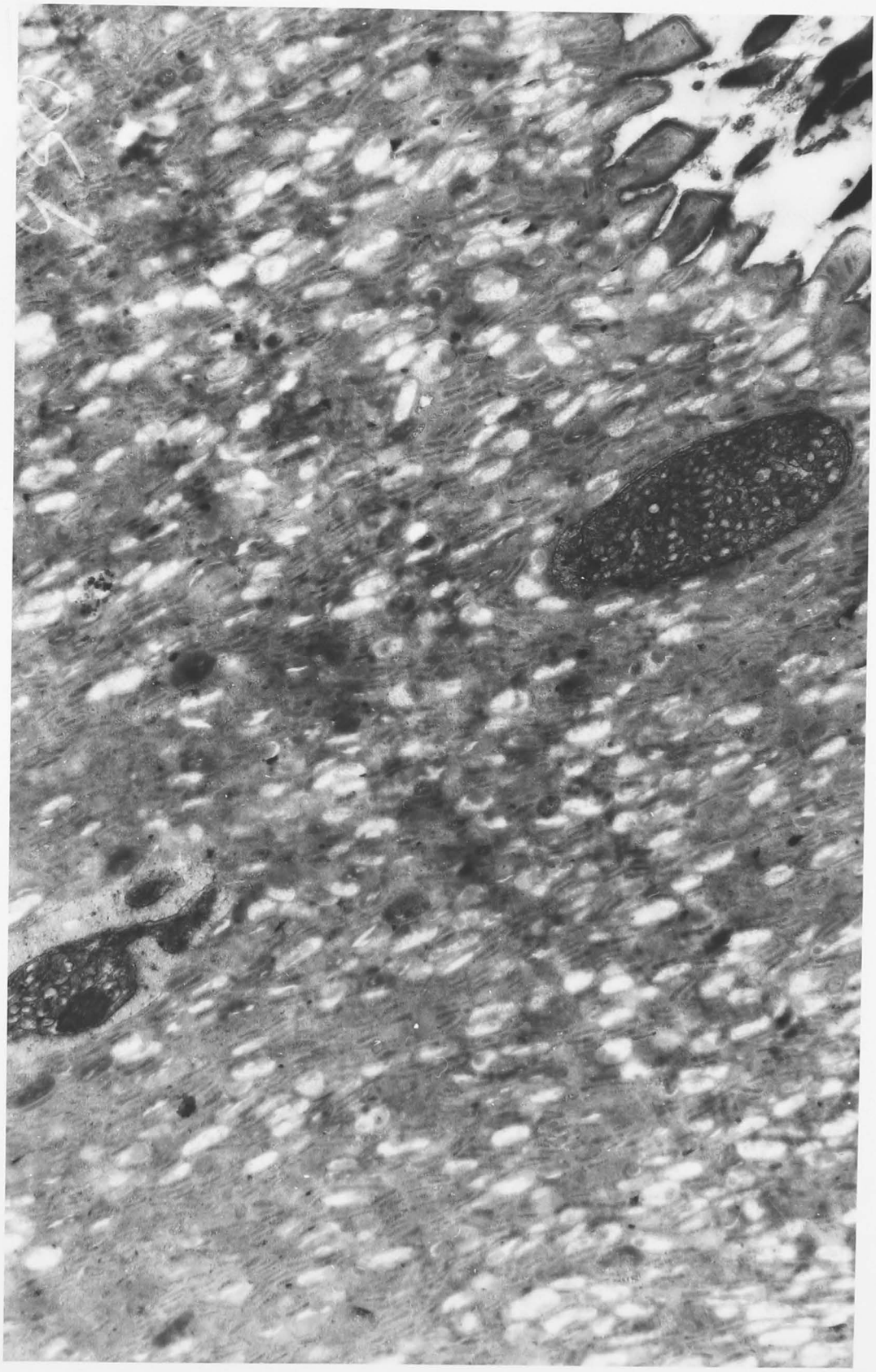


FIGURE 34

Two packets of granules in distal cytoplasm.
x40,000



formed in the perinuclear cytoplasm of the tegumental cell.

Structures shown in Fig. 38 were occasionally seen in the distal cytoplasm. They are 1.5 μ approximately in diameter, resemble sections of nuclei, but their true nature is not known.

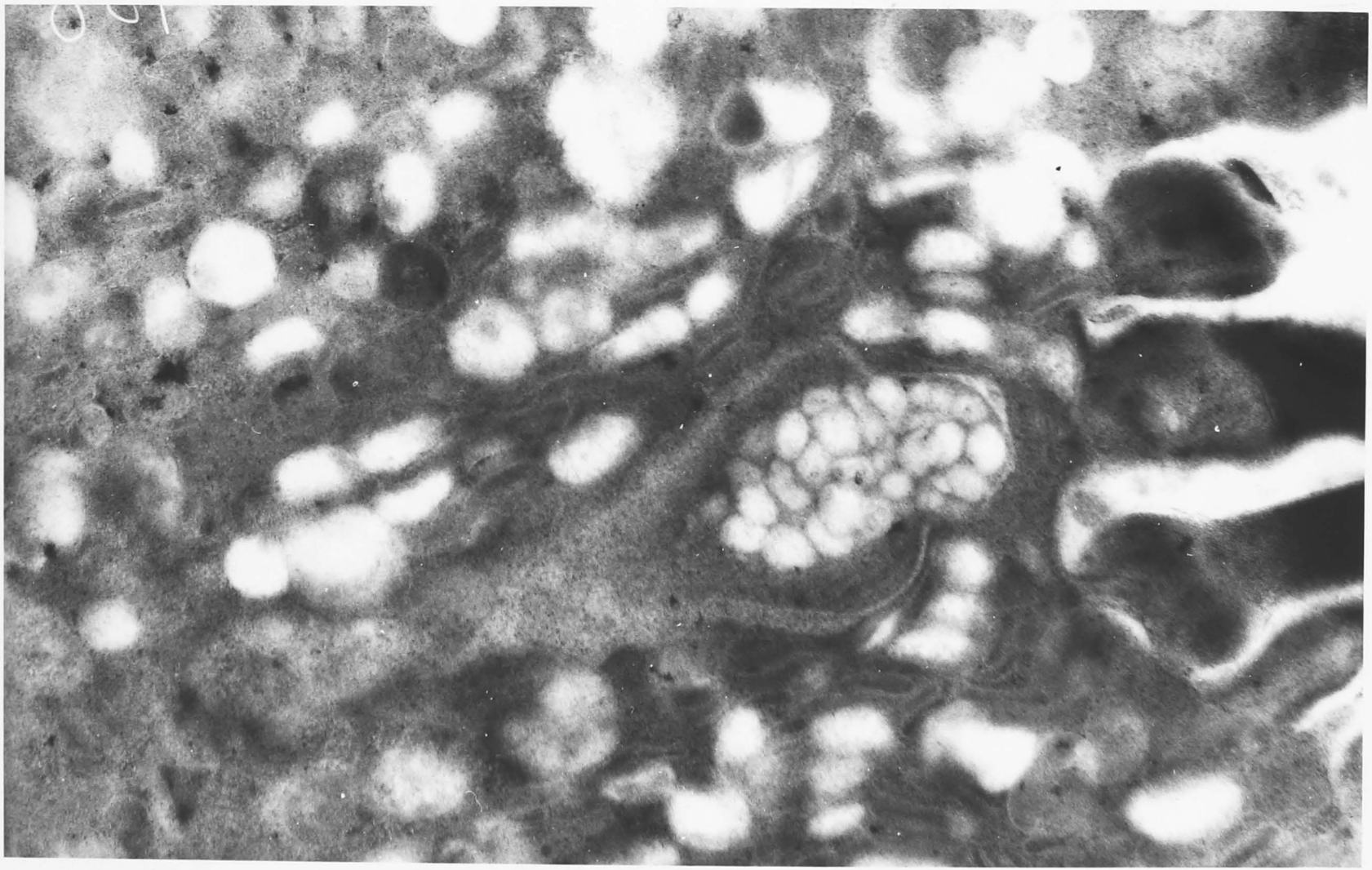
DISCUSSION

It is clear from various studies of the cestode tegument that the tegument can no longer be regarded as a tough inert cuticle which serves only to protect the worm from the digestive enzymes of the host. In fact the tegument has been shown to be a layer of living cytoplasm which is involved in the active uptake of nutrients, and to be well adapted to such a function (see Lee, 1966 for a review).

The tegument of S. erinacei was seen to be limited externally by a plasma membrane 105 Å thick which is continuous over the entire surface and covers the microtriches. Yamane (1968), in his study of Diphyllobothrium (= Spirometra) erinacei, made the same observation. The presence of an outer plasma membrane in the tegument has been reported by a number of authors in their studies on the tegument of other species of cestodes (Rothman, 1963;

FIGURE 35

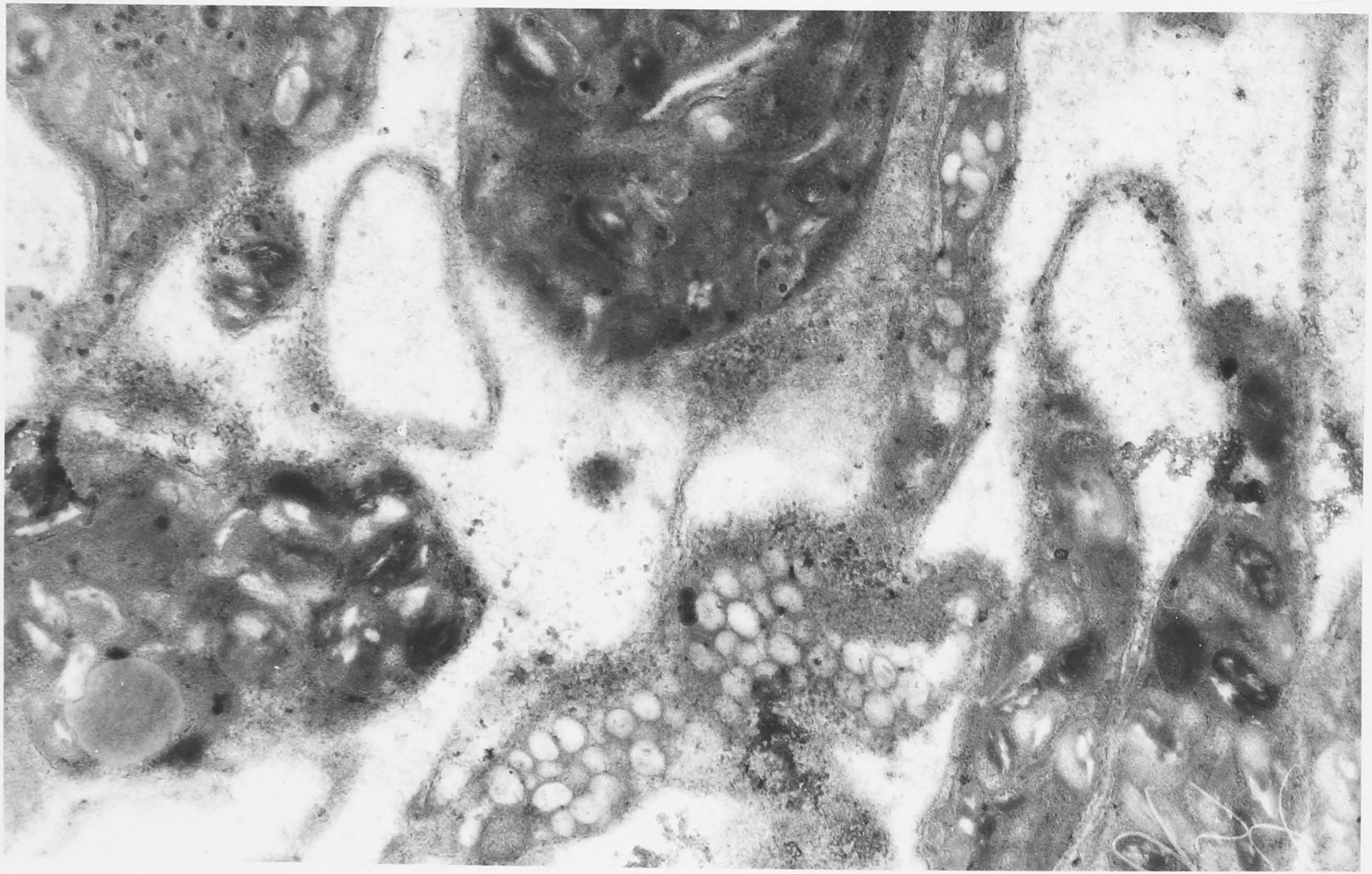
Packet of granules with proximal "trail" of amorphous material. x85,000



McCaig and Hopkins, 1965; Threadgold, 1965; Lumsden, 1966a; Morseth, 1966; Braten, 1968 a&b; Jha and Smyth, 1969). However, Jha and Smyth (1969) reported that the microthrix of Echinococcus granulosus is covered with a second outer membrane, about 115 Å to 145 Å thick, with gaps in it. This second outer membrane was not observed in the tegument of S. erinacei although a faint, broken, electron-dense coating can sometimes be detected over the external plasma membrane (see Fig. 15). This coating does not appear to constitute a continuous membrane and indeed is sometimes absent from the tegument of S. erinacei. Lumsden (1966a) in his study of Lacistorhynchus tenuis also described that a "fine, amorphous coating is applied to the outer surface of the unit membrane." McCaig and Hopkins (1965) noted that a dense precipitate or secretion covered the plasma membrane of Schistocephalus solidus after in vitro cultivation, giving it the appearance of a membrane. Braten (1968a) also noted the presence of an "amorphous coating" covering the surface membrane of Diphyllobothrium latum. Thus, at this stage, it is still uncertain if the tegument of S. erinacei does possess a second membrane covering the external plasma membrane, or if this is merely an artifact due to "precipitation of host body fluid or a secretion from the worm" as suggested by Braten (1968a).

FIGURE 36

Packets of granules in perinuclear cytoplasm of tegumental cell. Note irregular formation. x80,000



The microtriches of S. erinacei spargana are almost identical in size to those described by Yamane (1968). However, Yamane (1968) remarked that the "cylindrical proximal part" of the microthrix in the plerocercoid of S. erinacei constitutes only a small fraction of its length. However, in this study the proximal electron-transparent portion of the microthrix often measures up to 0.6μ long, thus constituting up to a quarter of the total length of the microthrix. Furthermore, the proximal portion appears rhomboid in cross-section and not cylindrical.

Yamane (1968) did in fact describe the proximal portion of the microthrix of S. erinacei as being "rhombic" in cross-section in the adult worm, as did several authors in their studies of other species of cestodes. For example, Jha and Smyth (1969) found that in Echinococcus granulosus the microtriches are rhomboid or polyhedral at the base of the shaft; in Dipylidium caninum, Threadgold (1962) described the base of the microthrix as "diamond-shaped" or square.

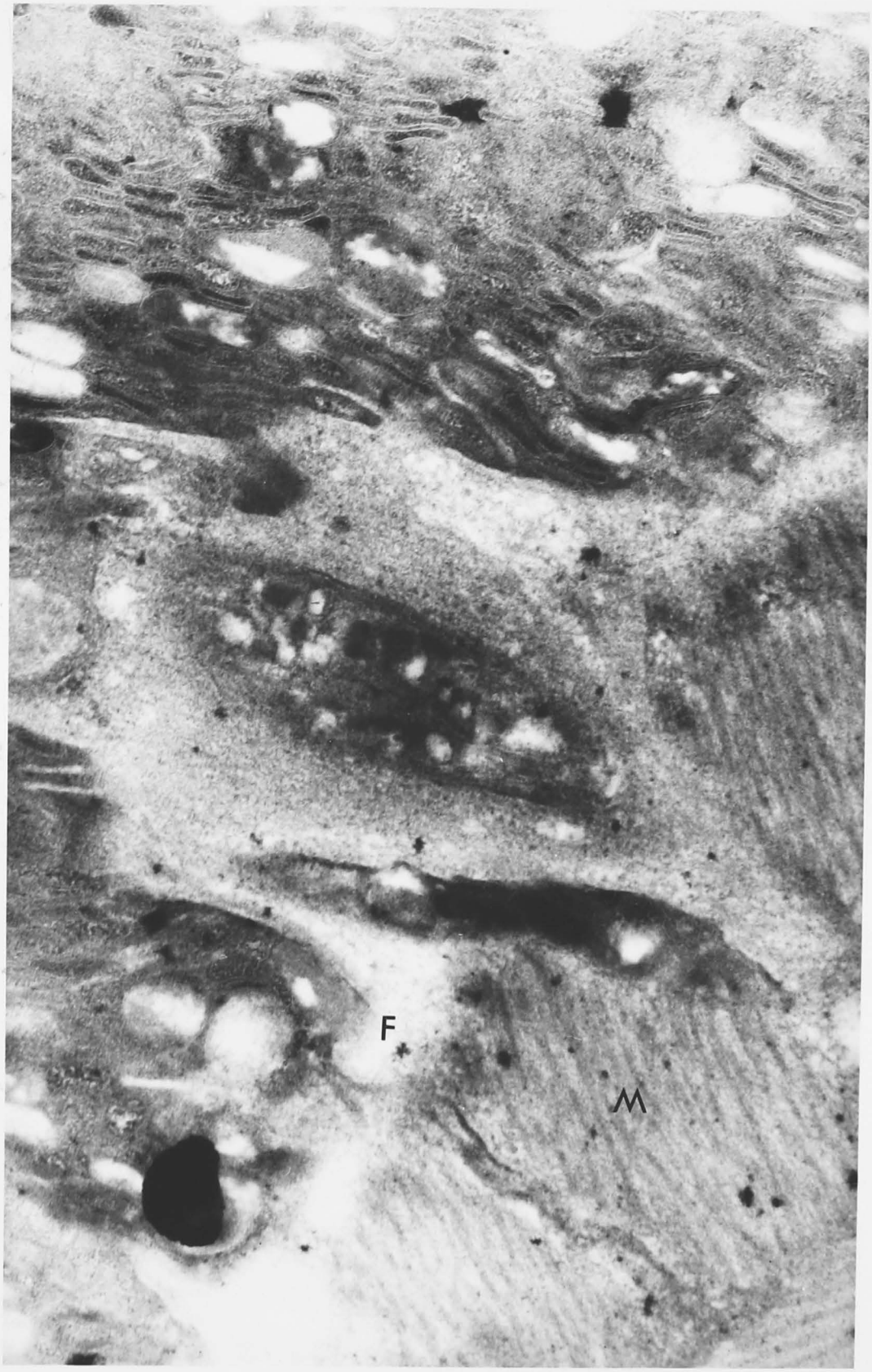
The division of the microthrix into an electron-dense distal portion and an electron-light proximal portion by a clear lucid zone, was also described by Yamane (1968).

FIGURE 37

Packet of granules at fibrous zone level.

List of abbreviations:
M-circular muscles; F-fibrous zone

x75,000

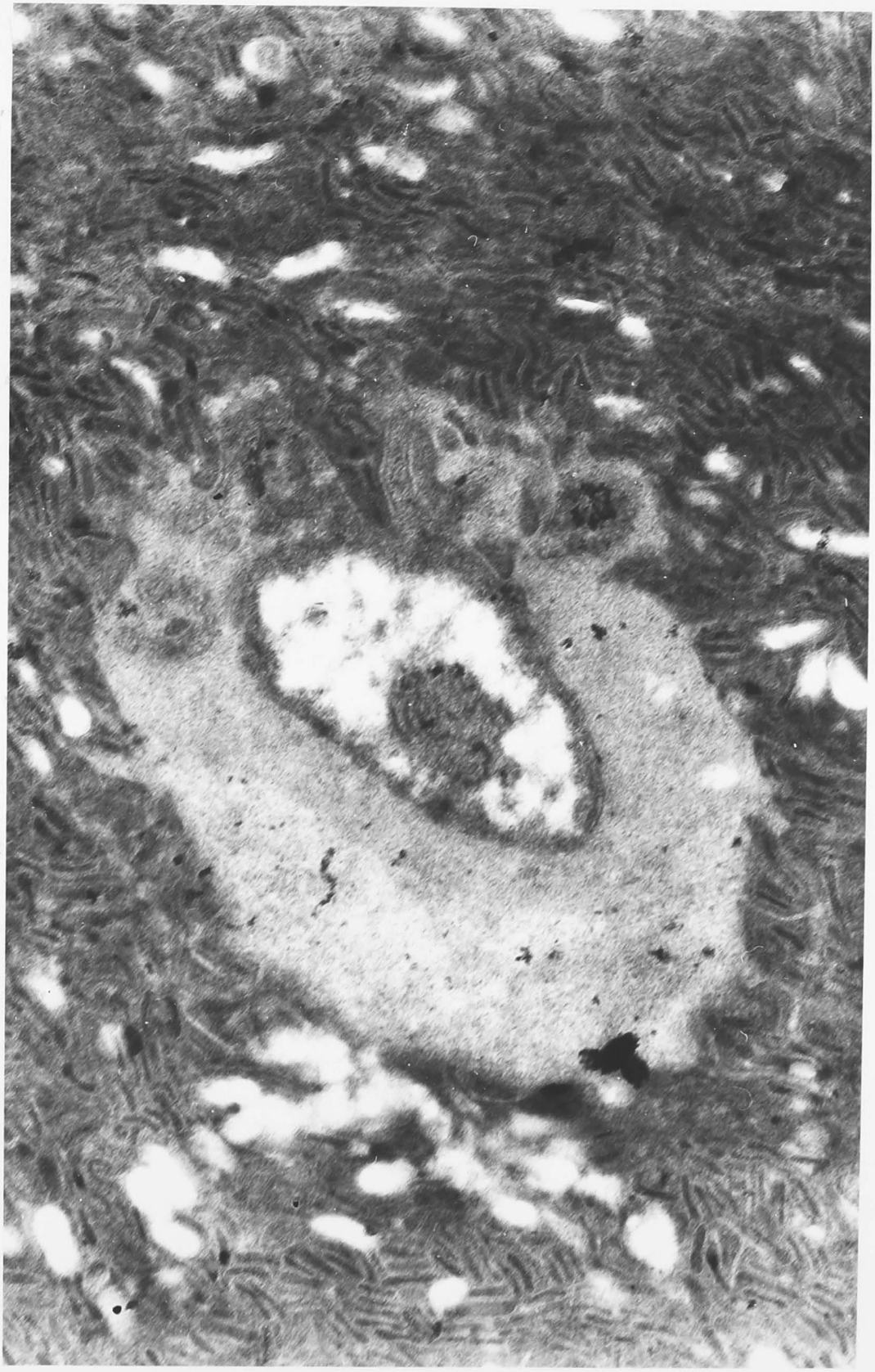

F
M

Morseth (1966) observed in Taenia hydatigena that this lucid zone is covered with two dark lines which are sometimes continuous, giving the lucid zone the appearance of a cross-section of a flattened hollow disc. Braten (1968a) called this a "membranous cap" and noted that the membrane of the cap is not continuous with the external plasma membrane since the electron-dense material from the distal portion of the microthrix can be seen to extend into the proximal part. This was also observed in S. erinacei where the electron-dense material extended peripherally down the proximal microthrix and into the matrix of the distal cytoplasm giving it the appearance of a "rootlet" at the base of a microthrix. The electron-micrograph in the paper by McCaig and Hopkins (1965) also shows these "rootlets" very clearly.

The electron-dense distal shaft of the microthrix of S. erinacei possesses a substructure of fine parallel longitudinal strands. Braten (1968a) noted a similar "lamellated substructure" in D. latum, and Lumsden (1966a) found "tubular or filamentous elements" within the distal microthrix of Hymenolepis diminuta. Morseth (1966) also found that the distal portion of the microthrix of Taenia hydatigena had a laminated appearance. Jha and Smyth (1969) showed that in E. granulosus a network of micro-tubules,

FIGURE 38

Structure probably through a nerve showing the nucleus of nerve cell. x70,000



which run, parallel to the longitudinal axis of the microthrix, is present in the distal shaft. These micro-tubules were revealed in cross-sections of the microthrix of E. granulosus at very high magnifications. It is probable that a similar network of micro-tubules is present in the microthrix of S. erinacei which impart the sub-structure of fine strands.

Several authors noted that finger-like projections of the distal cytoplasm, either bearing microtriches or not, sometimes appear in the tegument (Timofeev, 1964; Threadgold, 1965; Charles, Orr 1968). These were not observed in the tegument of S. erinacei in the present study or by Yamane (1968).

A number of membrane-bound inclusions have been observed by various authors in the distal cytoplasm of the tegument of cestodes. A number of different names therefore exist to describe these inclusions; among these are terms such as "vesicles, rhabdiform organelles, vacuoles, granules and rod-shaped bodies". In this study, the name vesicle refers to the ubiquitous empty membrane-bound structures, roughly circular in shape and measuring approximately 0.2μ in diameter. These vesicles are distributed uniformly throughout the distal cytoplasm. The

vacuoles, on the other hand, are much larger (from 0.6 μ to 1.0 μ in diameter), less common, and occur only near the external plasma membrane. These vacuoles may be pinocytotic vacuoles since they are enclosed by a membrane resembling the external plasma membrane; and in some cases connections with the exterior are encountered (see Figs. 18 and 19), and the membrane lining the vacuole is continuous with the external plasma membrane. Threadgold (1965) described similar large vacuoles in Proteocephalus pollanicoli as pinocytotic on the lesser evidence that "opposed plasma membranes can be observed in the region between the vacuole and the plasma membrane at the surface of the external level."

There is doubt as to whether a relationship exists between the smaller vesicles and the larger vacuoles. There was no evidence that the latter gave rise to the former or vice versa and it would seem justified at this stage to regard the vesicles and vacuoles as unrelated structures.

Yamane (1968) did not describe vesicles, but stated in discussion that numerous vesicles in the distal cytoplasm of S. erinacei were pinocytotic in origin. However, if this was so, then it would be reasonable to expect to

find involutions of the plasma membrane forming vesicles about 0.2μ in diameter, but so far, such involutions have not been detected.

The origin and function of the vesicles, as well as the electron-dense disc-like bodies, are unknown at this stage. Electron-dense disc-like bodies in S. erinacei were also noted by Yamane (1968) and Braten (1968a) noted similar structures in D. latum. Some of the disc-like bodies appear within the proximal portion of the microthrix (see Fig. 15) but they occupy much of the space within the distal cytoplasm.

Lyons (1969), in describing the distal cytoplasm of the tegument of the Cestodarian Gyrocotyle urna, observed the presence of vesicles and rod-bodies. These structures bear a striking resemblance to the vesicles and disc-like bodies of S. erinacei. She described the vesicles of G. urna as formed by the breakdown of the rod-bodies, and that they appeared to be secretory in nature. However, no evidence of such a relationship between the vesicles and disc-like bodies of S. erinacei was observed.

The lamellated bodies described by Braten (1968a) in D. latum were not seen in S. erinacei. Likewise the lipid droplets described by Threadgold (1965) in P. pollanicoli were not observed in the tegument of S. erinacei.

A number of canals have been described by various authors in the tegument of cestodes and here again, as with the cytoplasmic inclusions, different names have been used for them. There appear to be three main categories of canals which have been described. Firstly, there are those canals which extend up from the internal plasma membrane and project into the distal cytoplasm. The walls of these canals are essentially evaginations of the internal plasma membrane into the matrix of the distal cytoplasm. Yamane (1968) called these the "sub-cuticular canals" and described them as extending outwards from the "basement membrane" and ending as blind tubes some distance within the distal cytoplasm. They were found in the tegument of Diplogonoporus grandis as well as in S. erinacei, and those of D. grandis were associated with granule-containing vesicles. Morseth (1966) described similar canals in Taenia pisiformis, which ended blindly just below the external plasma membrane. These were referred to as "pore canals". (It is important to bear in mind at this stage the two terms "sub-cuticular canals" and "pore canals" since they are both used by other authors for totally different structures). Lumsden (1966a) found similar canals in H. diminuta which extended up to 0.75μ into the distal cytoplasm, ending as blind tubes.

These canals contained a finely fibrillar matrix continuous with the fibrous zone below the internal plasma membrane (Lumsden, 1966a). In the present study of the tegument of S. erinacei plerocercoids, these canals were not observed.

The internal plasma membrane of S. erinacei is however thrown into small infoldings into the distal cytoplasm. These do not constitute canals and never penetrate deep into the cytoplasmic matrix. Similar folds have been described by Threadgold (1962), Howells (1965), Braten (1968a) and Yamane (1968) in a number of cestodes.

The second category of canals concerns those which connect the distal cytoplasm of the tegument with the tegumental cells below. The walls of these canals are continuous with both the internal plasma membrane of the tegument and the plasma membrane surrounding the tegumental cells. Thus the cytoplasm of the tegumental cell is continuous, via cytoplasmic extensions, with the distal cytoplasm of the tegument, despite the intervening fibrous zone and muscle layers. These cytoplasmic extensions have been discribed by Threadgold (1962, 1964, 1965), Rothman (1963), Howells (1965), Lumsden (1966a), Morseth (1966) and Braten (1968a). Rothman (1963) called them "sub-cuticular canals". Similar structures were observed in

this study of S. erinacei and have been called cytoplasmic extensions.

The third type of canal opens from the outside into the distal cytoplasm. The walls of these canals are continuous with the external plasma membrane. Threadgold (1962) first described the presence of large canals in D. caninum opening to the exterior and extending down to, but not beyond, the internal plasma membrane, and they were termed "pore canals". These pore canals have also been described by Rothman (1963) who suggested that material from the exterior may go through to the "sub-cuticular canals" (cytoplasmic extensions) and directly into the parenchyma to be distributed. Threadgold (1962) however noted that the pore canals are never connected to the cytoplasmic extensions but end at the internal plasma membrane. In this study of S. erinacei, pore canals were not observed.

It can be seen then that the two types of tegument organelle described in the present study, namely (1) the pits whose openings are surrounded by a ring of cilia-like structures, and (2) packets of electron-transparent granules, do not appear to have been described in the cestode tegument previously.

There are indications that the tegument of cestodes may be involved in the secretion of macro molecular material, especially of enzymic proteins. Lumsden (1966b), using labelled amino-acids in auto-radiographic studies, indicated that there were high levels of protein synthetic activity in the tegumental cells and that the products were transported to the distal cytoplasm. He suggested that these products were "structural and non-mitochondrial enzymic proteins." These results were consistent with findings he made with the electron microscope (Lumsden, 1966a). He found that the Golgi-apparatus in the tegumental cell is surrounded by numerous membrane-bound vesicles which appear in intimate association with the ribosome-rich cisternae of the endoplasmic recticulum. This indicates that the tegumental cell is actively synthesising proteins. Furthermore, similar membrane-bound vesicles appear in the cytoplasmic extensions as well as in the distal cytoplasm, and they all possess a substructure indicative of quantum protein transport. Lumsden (1966 a&b) suggests that structural and enzymic proteins are synthesised in the tegumental cells and transported in quanta into the distal cytoplasm. It is, however, not known if this material is ultimately released at the surface.

Smyth (1969) suggested that vacuoles described by various authors, which open at the external plasma membrane to the exterior, may not necessarily be pinocytotic in nature. This could also apply to the vacuoles in S. erinacei referred to earlier. Another possible interpretation is that they may be indicative of the release of (enzymatic ?) macro molecular material from the tegument to the exterior. Howells (1965) briefly described "membrane-bound droplets" being extruded from the "surface membrane" of Monieza expansa. He also indicated that these may be associated with similar "granule-filled ovoid bodies which concentrate just below the cuticle surface."

The view that macro molecular material is secreted from the tegument of cestodes appears to receive support from the new types of tegument organelle described here for S. erinacei. It appears that material is secreted from the packets of electron-transparent granules in the form of membrane-bound granules about 0.06μ to 0.08μ in size. The granules enclosed in these packets are approximately the same size as the scattered "membrane-bound vesicles" described by Lumsden (1966a) in the tegument of L. tenuis. However, the "thickened inner cortex" of Lumsden's vesicles were not clearly observed in the granules of S. erinacei although some do appear to possess an

electron-dense "cortex" (see Fig. 33). The presence of a thickened cortex in the vesicle is apparently indicative of quantum intracellular protein transport (Lumsden, 1966a). Some of the membrane-bound granules in S. erinacei appear to have substructures within them (see Fig. 33). These are also seen in the electron-micrographs of the vesicles of L. tenuis (Lumsden, 1966a).

An alternative interpretation of the nature of the packets of electron-transparent granules in S. erinacei is that they represent sections through a nerve. Morseth (1967) described the presence of vesicles in sections of the lateral nerve trunks of Echinococcus granulosus. These vesicles vary in type and size from 250 Å to 1,600 Å and resemble the membrane-bound granules of S. erinacei. Since these nerves extend into the tegumental surface and terminate at sensory endings, it is possible to observe transverse sections of them which may appear as packets of vesicles. Lyons (1969) in describing the sense organs of the trematode G. urna also mentioned nerve endings in the tegument which contain "characteristic electron opaque vesicles measuring 0.06μ across."

However, it would seem that their interpretation of this type of organelle as sections of nerves can be

discounted, in S. erinacei at least, for the following reasons. Firstly, no long continuous sections of membrane-bound granules have been observed in the tegument, only discrete oval packets. Secondly, the packets in S. erinacei appear to be completely filled with membrane-bound granules, unlike the situation described by Morseth (1967) in which the "vesicles" are loosely scattered within the matrix of the nerve cell. Thirdly, neither Morseth (1967) nor Lyons (1969) described the release of "vesicles" to the exterior. Fourthly, Morseth (1967) described very definite structures associated with the sensory endings at the surface. These included the distal process, basal body, rootlets and septate desmosomes within the body of the sensory ending. Lyons (1969) also described a cilium with a 9 + 2 fibrillar substructure associated with the nerve endings. None of these structures were observed at the surface of the tegument of S. erinacei where the packets of membrane-bound granules open to the exterior. Fifthly, the whole bulb of the sensory ending of E. granulosus described by Morseth (1967) occupies the entire width of the distal cytoplasm. In S. erinacei the "flask-shape" packets of membrane-bound granules represent less than one tenth of the depth of the distal cytoplasm. The structures shown in Fig. 38 could however represent sections of nerves.

The much more tempting interpretation of the packets of membrane-bound granules in S. erinacei is that they represent enzymic proteins which are transported in quanta to the outside of the tegument where they could possibly be involved in tissue penetration. If the granules are in fact proteolytic enzymes, then it would be logical to expect them to be bounded and enclosed by membranes so as to prevent lytic action from occuring before the enzymes are released. Since more irregular packets of membrane-bound granules are also seen in the perinuclear cytoplasm of the tegumental cell, the granules may be synthesised in the tegumental cells and then transported as a discrete packet through the cytoplasmic extensions into the distal cytoplasm and eventually released at the surface.

To test such a theory, autoradiographic studies with labelled amino-acids similar to those made by Lumsden (1966b) at the light microscope level must be carried out at the electron-microscope level. However, although such studies may demonstrate movement of protein from the tegumental cell region to the exterior, the co-relation of this with proteolytic activity may prove to be very difficult since available techniques for the demonstration of proteases at the light microscope level would not be applicable at the electron-microscope level.

It should be noted also, that such studies might indicate whether the reverse of what is postulated occurs -- namely that the packets of granules form at the exterior, move down through the distal cytoplasm, and enter the tegumental region. The sunken pits, the other type of organelle described, would appear to be more permanent features than the packets of granules described above, since each has a ring of cilia-like structures around its opening to the exterior.

The origin of the closely packed electron-dense granules at the base of the pit organelle is not known. Again, material derived from the electron-dense granules, seem to be secreted from the organelles, mainly in the form of membrane-bound granules which resemble those released from the packets of granules. The fact that these organelles are located only near the tip of the scolex may explain why they have not been detected previously -- other workers may have used more distant regions of the strobila for their studies. The pits, like the packets of granules, do not bear any resemblance to sensory structures described in other cestodes. However, because of their location and the presence of the cilia-like structures, it seems reasonable that their involvement in sensory mechanisms should not be discounted at this stage.

Further discussion of the significance of the secretion of macro molecular material from the tegument of S. erinacei is given in Chapter 6.

CHAPTER 5

PROTEOLYTIC ACTIVITY IN THE SPARGANUM OF SPIROMETRA ERINACEI

INTRODUCTION

As the scolex of the sparganum of Spirometra erinacei possesses weakly developed bothria rather than hooks and powerful suckers, the process of penetration through the intestinal wall is unlikely to be achieved by mechanical action of the scolex alone. It would appear rather that mechanical action would be of secondary importance and that the initial breakthrough is achieved by the action of secretions of the parasite since it takes such a short time for a sparganum to penetrate through fairly thick bands of intestinal muscle and a layer of collagen.

As noted in Chapter 3, prominent gland cells in the sparganum, which might be involved in penetration of the intestine, were not detected. However small organelles were resolved in electron microscope studies described in Chapter 4 and it was suggested that these may secrete a proteolytic enzyme(s), or possibly a battery of different sorts of enzymes which, acting in conjunction with the muscular activity of the scolex, might account for rapid

penetration. This chapter describes investigations which were carried out to establish whether a proteolytic enzyme is present in the scolex, and, if so, whether activity might be correlated with morphological features discussed in Chapter 4.

METHODS AND MATERIALS

(a) Biochemical demonstration of proteolytic enzymes by colorimetric assay

The procedure followed in an attempt to detect proteolytic enzymes in the sparganum was a biochemical assay, based partly on the method by Anson (1938) and partly on Rosen (1957) with the following modifications. Unlike Anson the substrate used here was casein (B.D.H.) instead of haemoglobin. Although casein is not a reproducible substrate, i.e. different batches may be digested at different rates by the same protease, this factor did not affect the results of the experiment described here since only a qualitative demonstration of proteolytic activity was sought. All colorimetric readings were as in Rosen's modified Ninhydrin colorimetric analysis.

Approximately 20 mg wet weight of freshly collected spargana were homogenised in a manual glass homogeniser

with 2 ml of acetate buffer.

Two separate sets of readings were made with samples of homogenate. A third set of readings were taken with samples of homogenate which were heated to 100°C for 15 min to denature any enzymes which might have been present. This acted as a control. To eliminate spurious readings which might be obtained because of bacterial contamination of the incubation medium, other appropriate controls (all components but lacking worm homogenate) were carried out.

(b) Histochemical demonstration of proteolytic enzymes by gelatin-silver film substrate

The method used in an attempt to detect proteolytic enzymes in the scolex of the sparganum histochemically, was based on that given by Adams and Tuqan (1961). The spargana used for these experiments included scoleces which had recently penetrated the gut of mice as well as unpenetrated scoleces. Both fresh spargana and those fixed in 4% formol-saline for 24 hr at 4°C were used. All scoleces were washed several times in freshly prepared Ringer's before use.

Frozen sections of the spargana were then cut at 16μ on a cryostat and mounted on Ilford photographic plates

(N50 emulsion), exposed and developed to a density of 3.0. A drop of the Ringer's, which was used to wash the scoleces, was placed on the plate some distance from the sections. This acted as a check against digestion due to bacterial contamination. For the negative control, the scolex was heated to 100°C in Ringer's for 10 min and sections were cut and mounted on another part of the same photographic plate. The plates were then incubated at 37°C in a humid atmosphere. Four sets of plates were removed after ½ hr, 1 hr, 1½ hr, and 2 hr respectively, from the incubator. The plates were then immediately dehydrated through the alcohols, cleared in xylol and mounted in Canada balsam. Viewing under the microscope was done only after mounting to prevent the melting and "crazing" of the plate emulsion. This occurred if a damp photographic plate was placed over the high-intensity light beam necessary to detect digested areas of the plate.

RESULTS

(a) Biochemical demonstration of proteolytic enzymes in spargana

The readings from the colorimetric analysis were plotted as a graph to show the relationship between

FIGURE 39

Biochemical demonstration of proteolytic enzyme(s) in spargana.
Graph represents readings of colorimetric analysis, showing relationship between incubation time and colour yield. Solid lines represent increasing amounts of amino-acids liberated, indicating digestion; two test runs are plotted. Dotted line represents control run; no digestion recorded due to heat treatment.

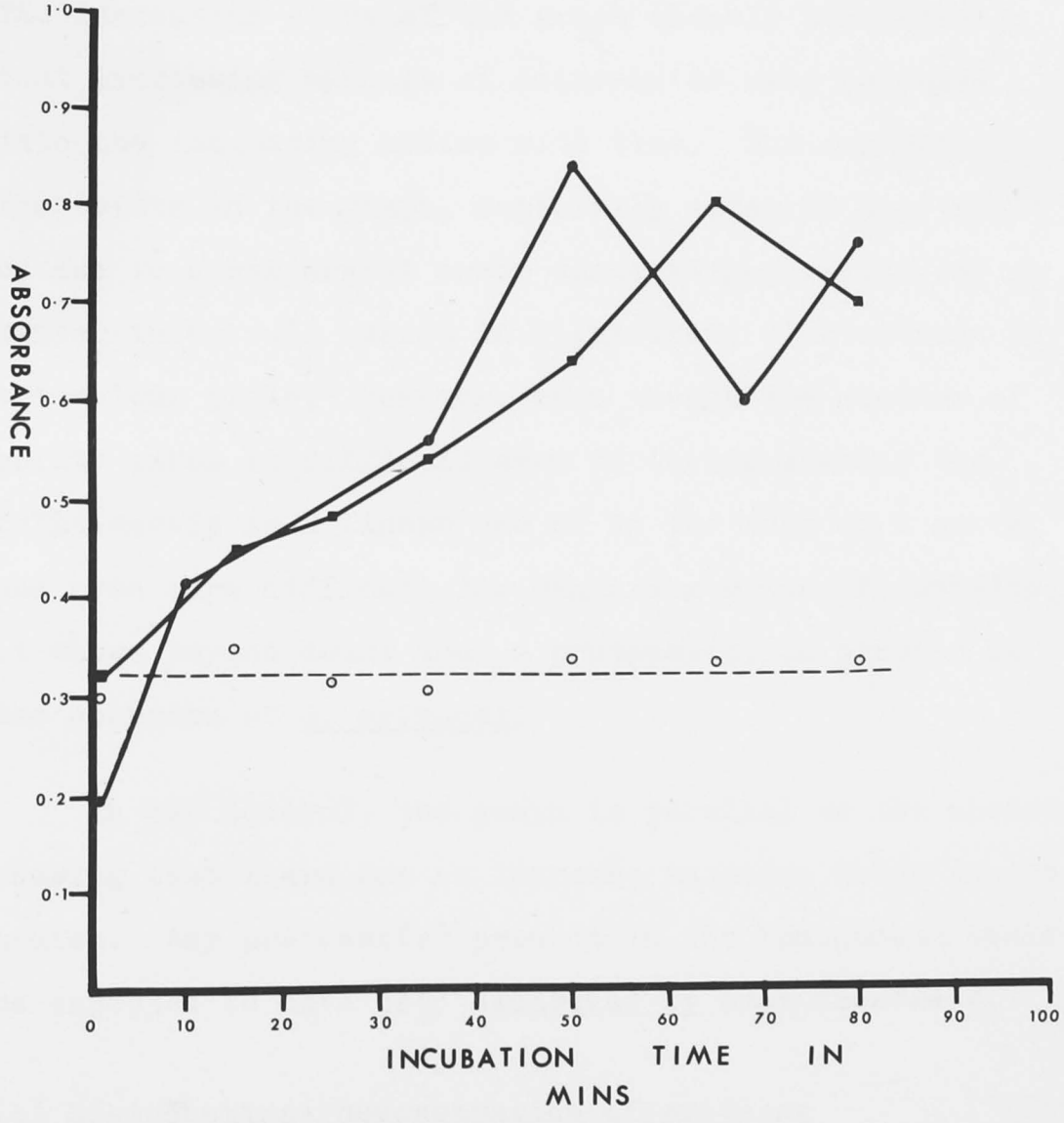

ABSORBANCE
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
10
20
30
40
50
60
70
80
90
100
INCUBATION TIME IN MINS

FIGURE 40

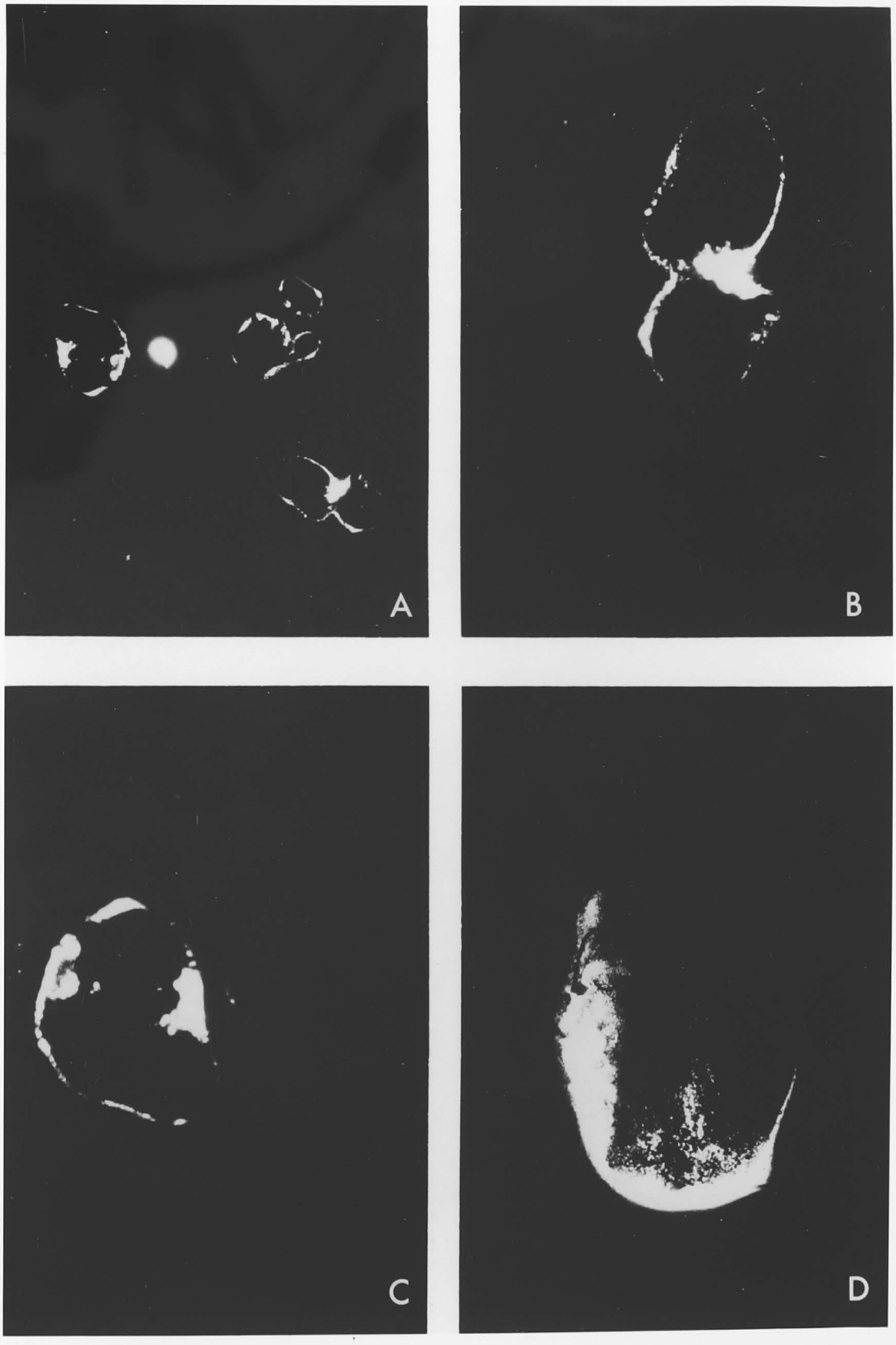
Test for proteolytic enzyme(s) by incubating sections on blackened photographic plates.

A - L.P. showing several sections on plate. Sites of enzyme(s) show up as clear digested areas. Note that enzyme(s) is present only in tegumental region; no digestion in medullary region of scolex. Clear round area seen is due to plate imperfection. (1 hr incubation) x15

B - As above, at higher magnification. x40

C - As above in B. x40

D - Area of digestion increased possibly due to autolysis or longer (2 hr) incubation. x40


A
B
C
D

seen in Fig. 40,A,B, and C, the areas of proteolytic activity occurred only in the region of the tegument. The optimum incubation time in this case was 1 hr. After 2 hr of incubation, however, a much larger area of digestion was evident. The digested area may even spread over to regions beyond the circumference of the sections (see Fig. 40,D). This was probably due to the combined effects of the protease(s) and autolysis in the tissues since no such generalised digestion occurred after 1½ hr. Sections of both penetrated as well as unpenetrated scoleces gave similar results.

Sections of fixed spargana did not digest the photographic plate, suggesting that the protease(s) in the sparganum was labile, its activity destroyed by formaldehyde fixation. The controls were negative, showing that digestion did not result from contamination of the incubation medium and the protease(s) was thermolabile.

DISCUSSION

Proteolytic enzymes have been previously reported in other cestodes such as D. latum, E. granulosus and Taenia spp. (Read and Simmons, 1963) although it is

not known if these are intracellular or extracellular enzymes. As described above, proteolytic enzyme(s) were detected biochemically in homogenates of S. erinacei spargana, and the site of these enzyme(s) were located by incubating sections of the worm on exposed photographic plates. The results indicated that (i) proteolytic enzyme(s) is present in the sparganum of S. erinacei; (ii) the enzyme(s) is thermolabile and can be destroyed by formalin fixation; (iii) the enzyme(s) is closely associated with the tegument of the scolex.

Although the presence of a proteolytic enzyme(s) is established, its role in penetration is, at present, far from clearly understood. Because of the poor resolution obtainable with the test for proteolytic activity it is not known whether the proteolytic enzyme(s) is extracellular, intracellular, or bound to the outer limiting layer of the tegument. If the enzyme(s) is extracellular this would mean then that it is secreted by the sparganum to the exterior and thus possibly be involved in sparganum penetration. If the enzyme(s) is intracellular, on the other hand, e.g. in pinocytotic vacuoles, it would not be involved in penetration but rather concerned with intracellular digestion. If

membrane-bound, the proteolytic enzyme(s) could be involved in "membrane (contact) digestion". This will be discussed in greater detail later (Chapter 6). It is also possible that other enzymes (e.g. esterase) are associated with the penetration process. However, in the case of esterase, activity was not clearly established and further experimentation with esterase techniques is necessary (see Chapter 3). The only other enzyme demonstrated, alkaline phosphatase, is not likely to have a pronounced lytic role on the host, but is more probably concerned with absorption (Halton, 1967).

An attempt was made to determine if the proteolytic enzyme(s) was extracellular. Live spargana of S. erinacei were incubated on exposed photographic plates in a humid atmosphere at 37°C; the spargana used were either taken directly from the natural host or immediately after penetration through mouse intestine. Some spargana were also placed in small dishes of Ringer's solution at 37°C, stimulating them to detach their scoleces from the rest of the strobila before placing the scoleces on exposed photographic plates. Drops of Ringer's solution, used as the incubation medium, were

also incubated on the plates as controls. In all cases, no digestion of the plates was observed even though the times of incubation were similar to those used for sections. Although negative results do not unequivocally prove that the proteolytic enzyme(s) is not extracellular, they would appear to indicate that proteases are not secreted to the exterior in any significant quantities.

If the proteolytic enzyme(s) is not secreted extracellularly, it would still have to be determined whether the enzyme(s) is intracellular or bound to the outer limiting layer of the tegument; knowledge of this would suggest a likely function of the enzyme(s). Differential centrifugation of the sparganum homogenate, for example, could be carried out and membranous cell organelles and supernatant tested independently for proteolytic activity by biochemical methods. Unfortunately, such as experiment, a logical extension to those described, has not yet been carried out.

It is worth noting that no attempt has yet been made to find out if the proteolytic enzyme(s) is present not only in the scolex of the sparganum, but also in the rest of the strobila. However, since the strobila is

discarded before penetration, it is quite probable that the proteolytic enzyme(s), even if it is present in this region, is not involved in the penetration process.

An attempt was made to determine whether a collagenase is present in the sparganum scolex of S. erinacei, because it is known that a relatively thick layer of collagen is present in the mouse intestine. This was carried out by incubating live spargana as well as homogenates on a film of rat tail collagen prepared as indicated by Gross and Kirk (1958) but no areas of digestion were observed. However, negative results such as these do not unequivocally prove the absence of a collagenase in the sparganum.

CHAPTER 6

GENERAL DISCUSSION

From the results of histochemical studies reported in Chapter 3, no clearly demonstrable glands were evident in the scolex of spargana of S. erinacei. Most of the significant histochemical activity in fact could not be associated with any organs within the sparganum. The tegumental cells, however, are rich in RNA indicating they undergo active protein synthesis. Moreover, the tegument is also strongly PAS-positive. Seen in relation to the fact that proteolytic activity was also demonstrated in the same region, the tegument of S. erinacei assumes added functional significance.

Furthermore, there are indications that material, probably synthesised in the perinuclear cytoplasm of the tegumental cell, then brought up to the distal tegument, may be released from the sparganum scolex of S. erinacei. Electron-microscopic evidence that this may occur has been discussed in Chapter 4. Although extracellular proteolytic enzyme(s) has not been demonstrated, it may be present -- but in quantities not detectable by the methods described above -- and indeed associated with

the ultrastructural features described in Chapter 4.

The data accumulated so far in this study of the sparganum of S. erinacei makes it possible to propose a tentative model for the mechanism whereby the sparganum penetrates the intestinal wall of its intermediate host.

Smyth (1969) has suggested that membrane digestion may occur at the host-parasite interface. Ugolev (1965) in his discussion on membrane digestion in parasites has also suggested that the tegument of some parasites bears "a resemblance to the inverted pieces of small intestines" where membrane digestion occurs. In the process of membrane (contact) digestion, the final break down of material occurs on the surface of membranes to which the digestive enzymes are attached. The concept of membrane digestion has generally been associated with nutrition and absorption of food material and was in fact first put forward to explain certain phenomena associated with the mucosal surface of the small intestine (Ugolev, 1965).

Smyth, Miller and Howkins (1967) suggested that the same process may occur at the host-parasite interface between the scolex surface of Echinoccocus granulosus

and the host duodenum. Jha and Smyth (1969) suggested, on the basis of electron-microscopic findings, that since the microtriches of E. granulosus are covered with what appear to be secretory membranes with "globular" substructures, digestive action may take place only when the parasite surface is in intimate contact with the substrate (membrane digestion). This view is further supported by the fact that the outer limiting membrane of E. granulosus is similar in structure and dimensions to mitochondrial membranes which are associated with enzymatic functions. Results of physiological studies on the tegument of H. diminuta, H. microstoma and M. expansa (Taylor and Thomas, 1968) suggest that membrane digestion is also operative in these cestodes. Thus, membrane digestion could be a wide-spread phenomenon among cestode teguments, and the proteolytic enzyme(s) in the tegument of S. erinacei may well be bound to the external limiting membrane and be involved in this process.

Although all the evidence so far accumulated about membrane digestion has characterised it as a phenomenon closely associated with absorption of food material, possibly in certain cases it may be involved only in the

process of digestion and not necessarily in the absorption of food substances. The occurence of membrane digestion appears to be fairly wide-spread, and is found both in vertebrates and invertebrates as well as in micro-organisms such as bacteria and yeast cells. Ugolev (1965) in fact proposed that, in the process of evolution, membrane digestion may be found in other types of cells in higher animals, not necessarily associated with purely "digestive-absorptive" functions. It is thus not inconceivable that membrane digestion may have evolved to function in purely digestive roles such as tissue digestion by migratory parasites and in tissue penetration by parasites.

If membrane digestion occurs at the surface of the S. erinacei sparganum scolex, which is in close contact with the duodenal epithelium of the host as the sparganum pushes against the duodenal wall, initial rupture of the epithelium might occur quickly. Then, the process of membrane digestion by the sparganum, in conjunction with autolysis brought about by the action of lumen and gut intracellular enzymes, could result in rapid penetration. In this case even the mechanical action of the muscular scolex, which may be of little importance in the primary

rupture of the epithelium, may become important in facilitating autolysis.

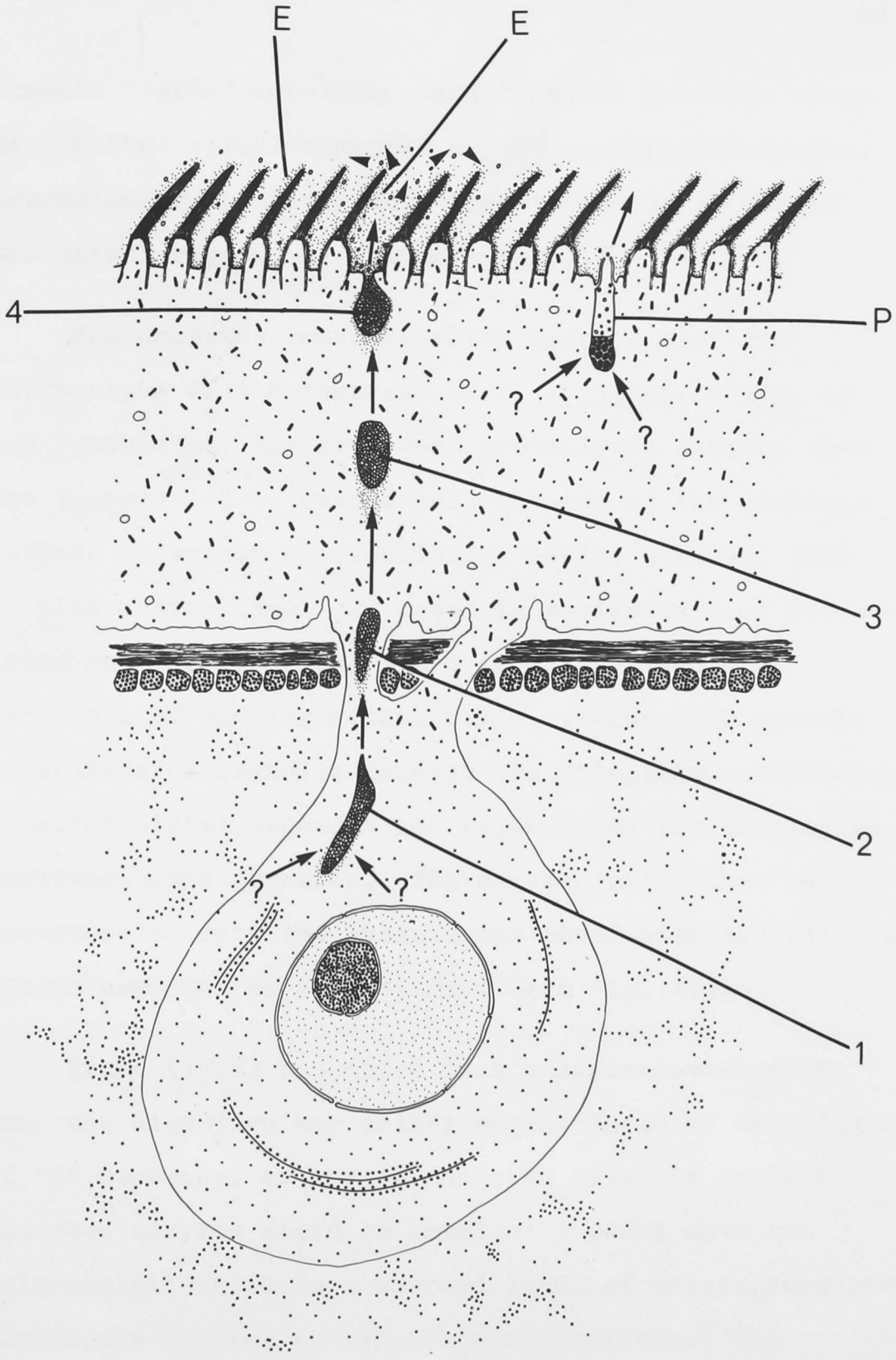
This could explain how a soft-bodied organism like the sparganum, with no apparent specialised organ for penetration, can penetrate through the gut wall in as short a time as 10 min. It would also explain why prominent glands, secreting material through well defined ducts, were not found in the sparganum of S. erinacei, since large amounts of extracellular enzymes need not necessarily be secreted. That the latter does not occur is substantiated by the fact that attempts to demonstrate the presence of extracellular enzymes have failed.

The presence of proteolytic enzymes in the tegument of the scolex of the S. erinacei sparganum, together with large amount of RNA in the tegumental cells, suggests that the tegument of the sparganum may be both the site of enzyme synthesis and enzyme action. This view receives some support from the work of Lumsden (1966 a&b). He found that following incubation in labelled amino-acids, proteinaceous substances are in fact released through the tegument by some parasites and he suggested that this may be associated with the "vesicles" he found in electron-microscopic studies of the tegument of the same parasites.

FIGURE 41

Diagram explaining the proposed hypothetical model for the mechanism of penetration by the sparganum.

1 - Proteolytic enzyme(s) is synthesised in the perinuclear cytoplasm of the tegumental cell in the form of a packet of enzymic granules.
2 - Packet of enzyme(s) moves upwards through cytoplasmic extension into distal cytoplasm of the tegument.
3 - Packet of enzyme(s) continues upwards through distal cytoplasm of the tegument.
4 - Packet opens at surface, liberating the enzymic granules.
E - Enzyme(s) attached on glycocalyx at surface of tegument; here the enzyme(s) is involved in membrane digestion when the surface of the sparganum comes in contact with the mucosal surface of the host intestine.
P - Pit organelle. The function of this organelle is enigmatic.


E
E
4
P
?
?
3
2
?
?
1

Lumsden (1966a) concluded that "enzymic proteins occuring in the cuticular matrix may be synthesised in the subcuticular cytoplasm then transported in quanta to the apical regions of the integument."

The membrane-bound granules seen in electron-micrographs of the tegument of S. erinacei, which, it was speculated, may represent proteolytic enzymes that are transported in quanta and released at the tegument surface to become extracellular, could, however, have an alternative fate. It could be envisaged that proteolytic enzymes are synthesised in the perinuclear cytoplasm of the tegumental cells, transported upwards as packets of granules through the cytoplasmic extensions into the distal tegument and released at the surface as membrane-bound granules. The enzymes might then be incorporated onto the surface and bound with the external plasma membrane of the tegument (see Fig. 41).

Crane (1968) has noted that enzymes involved in membrane digestion may either be intrinsic to the structure of the membrane, or may be adsorbed onto the surface. Adsorbed enzymes would be chemically bound onto the "glycocalyx" which is a charged layer of mucopolysaccharide supposedly present on all membrane surfaces. The

"glycocalyx" corresponds to the PAS-positive layer on the tegument of S. erinacei seen in light microscopy and also to the thin electron-dense amorphous coating seen on the distal plasma membrane of the tegument under the electron-microscope (as also suggested by Lumsden, 1966a; Smyth, 1969). The proteolytic enzymes, synthesised in the perinuclear cytoplasm and secreted to the surface of the tegument could then be bound on to the "glycocalyx" and be active in membrane digestion when the sparganum comes in contact with the mucosal surface of the host duodenum, and thus initiate penetration.

Mueller (1961) has found that incubating spargana of S. mansonoides in immune sera results in a precipitate forming around the scolex. Thus, the precipitate may be a manifestation of an antigen-antibody reaction against antigenic determinants at the surface of the sparganа. These antigenic determinants could themselves be parts of membrane-bound histolytic enzymes at the tegumental surface, similar to those suggested as being present in S. erinacei, and associated with penetration by spargana into the intermediate host.

Although such a theoretical model appears attractive

in explaining the mechanism of penetration, it is largely speculative. However, it does provide a framework for further experimental studies on a phenomenon which is undoubtedly subtle and still remains largely enigmatic.

SUMMARY

A study was made on the sparganum of Spirometra erinacei in an attempt to determine the mechanism involved to account for the penetrative ability of the sparganum.

Using mice as experimental hosts, the sparganum of S. erinacei was shown to penetrate the duodenum, taking only 5-10 min to complete the process. Proteolytic enzyme(s) was shown to be present in the scolex of the sparganum, closely associated with the tegument. The enzyme(s) did not appear to be present in regions other than the tegument. Morphological and histochemical studies showed that the sparganum of S. erinacei, unlike plerocercoids of some other pseudophyllidean cestodes, do not possess clearly defined glands or gland cells in the scolex. The presence of large amounts of RNA in the tegumental cells, however, indicates that a high level of protein synthesis occurs in this region. The tegument was shown to be rich in protein and strongly PAS positive.

Studies with the electron-microscope demonstrated the presence of two types of tegumental organelle which

do not appear to have been previously described in cestodes. It is suggested that these organelles are involved in the release of macro molecular material from the tegument of S. erinacei. With one type of organelle, it was speculated that granules may be synthesised in the perinuclear cytoplasm of the tegumental cells, transported upwards to the distal cytoplasm as packets of granules, then released at the surface and adsorbed onto the external plasma membrane of the worm. A theoretical model for sparganum penetration is proposed which considers that these granules consist of proteolytic enzyme(s); these are adsorbed onto the outer limiting membrane of the tegument and membrane digestion of the epithelium lining of the duodenum of the host is the primary event in penetration.

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